SEXUAL COMMUNICATION IN MICE: LINKING MALE SIGNALS, FEMALE LEARNING AND ADULT NEUROGENESIS

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"I am among those who think that science has great beauty. A scientist in his laboratory is not a mere technician; he is also a child confronting natural phenomena that impress him as though they were fairy tales."

Marie Curie 1867 - 1934

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ABBREVIATIONS

Ab	Antibody
Acb	Nucleus Accumbens
AMY	Amygdala
AON	Anterior Olfactory Nucleus
AOS	Accessory Olfactory System
BNST	Bed Nucleus of Stria Terminalis
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
CAG	Chrome-Alum Gelatin
CPP	Conditioned Place Preference
Da	Dalton
DCX	Doublecortin
DG	Dentate Gyrus
Dlx	Distal-Less
DTT	Dithiothreitol
EC	Entorhinal Cortex
ESP	Exocrine-gland Secreting Peptides
FPR	Formyl Peptide Receptor
GC-D	Guanylyl Cyclase D
GCL	Granule Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GG	Grüneberg ganglion
HC	Hippocampus
HPA	Hypothalamo-Pituitary-Adrenal Axis
HTH	Hypothalamus
IHC	Immunohistochemistry
MHC	Major Histocompatibility Complex
MOB	Main Olfactory Bulb
MOE	Main Olfactory Epithelium
MOS	Main Olfactory System
MPA	Medial Preoptic Area
MUP	Major Urinary Protein
NeuN	Neuronal-Nuclear Antigen
OB	Olfactory Bulb
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
OT	Olfactory Tubercle
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PC	Piriform Cortex
PCA	Principal Components Analysis
PCNA	Proliferating Cell Nuclear Antigen
PSA-NCAM	Polysialic Acid-Neural Adhesion Molecule
RMS	Rostral Migratory Stream
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SGZ	Sub Granular Zone
SO	Septal Organ
SVZ	Sub Ventricular Zone

TAAR	Trace Amine-Associated Receptor
TH	Thalamus
USV	Ultrasonic Vocalisation
VNO	Vomeronasal Organ
VP	Ventral Pallidum
VTA	Ventral Tegmental Area

Sexual communication in mice: linking male signals, female learning and adult neurogenesis.

For many species sexually selected signals are essential for communication between conspecifics. These signals produced by males are attractive to females and may reliably reflect competitive ability, health status and genetic identity. There is high diversity in the types of auditory, visual and olfactory signals that males produce, and males of many species invest in multiple signals. In mice, adult males invest in scent-marks and ultrasonic calls as sexual signals. Ultrasonic calls are emitted during encounters with female conspecifics, primarily during pre-copulatory sniffing and investigation. As a result, this sexually dimorphic and individually variable behaviour has been suggested to serve a specific courtship function in mice. Scent marks are also important for communication with conspecifics and provide long lasting signals of identity, health status and competitive ability to potential mates. Both male signals are therefore important in sexual communication, but it is unclear whether scent marking and calling serve separate functions. To address this, male scent marking and calling was recorded in response to female odours, a female behind a mesh barrier, a direct interaction with a female or no female cues. Male signalling investment varied according to female cue type. Further, cues that elicited a high rate of calling differed from those that elicited a high rate of scent marking. Ultrasonic calls were primarily emitted in interactions suggesting an important role in direct courtship. By contrast, the greatest number of scent marks was deposited when males were not in direct contact with female conspecifics.

Consistent with the results obtained in the first experiment, scent marks are known to provide information to females in the absence of a male through long lasting cues present in urine. The distribution of scent marks also provides an honest signal of a male's competitive ability to defend its territory. Females are highly attracted to spend time near male scent marks and are subsequently more attracted to the owners of these familiar scent marks than to equivalent unfamiliar males. Male scent can also condition a preference for its location, stimulated by a specific sex pheromone present in male urine. These acquired memories of male scent mark locations and individual scent signatures are likely to be important in allowing females to be selective and approach preferred males when ready to mate. In a natural context, females investigate multiple

scent marks when territories of competing males are encountered. To reflect the complexity of scents encountered in a natural context and to assess how differences in male expression of specific scent components influence female spatial learning, conditioned place preference tests comprising multiple scents and locations were used. When multiple scents were presented simultaneously, females formed a learned preference for multiple locations of male but not female scents; darcin, a male-specific pheromone, was also required for male scent to condition a preference for its remembered location. Further, the relative amount of darcin influenced the strength of a remembered preference. The comparative composition of individual scents may therefore be important in determining female learned preferences for locations scent marked by competing males.

Male scent from dominant territory owners also stimulates neurogenesis in the hippocampus and olfactory bulbs of female mice, areas of the mammalian brain associated with spatial learning and olfactory processing. In hippocampal neurogenesis, neurons are generated and remain in their mature form within this same structure; by contrast, the germinal area for olfactory neurons is present in the forebrain and newly generated cells must migrate over long distances before being incorporated into existing circuits. To investigate whether scent components that stimulate spatial learning also stimulate neurogenesis in females, immature hippocampal neurons and proliferating cells in the forebrain were quantified in female mice exposed to different male scents or scent components. The results suggest darcin, a male specific MUP that stimulates spatial learning, also stimulates hippocampal and olfactory neurogenesis in females. This male pheromone therefore appears to play a role in the immediate behavioural response and the long-term neurological change associated with exposure to male scents.

CHAPTER 1: General introduction

1.1 SEXUAL SELECTION

When Darwin first published his work on natural selection in 1859, one of the major problems facing his theory was the existence of conspicuous male traits such as bright coloration or exaggerated ornaments. These characteristics appeared to reduce survival and should therefore be opposed by natural selection (Darwin, 1859). However, many of these traits not only exist in a variety of species, but they remain stable within populations. To solve this problem, Darwin (1859) postulated sexual selection as a process distinct from natural selection that "…depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring". Sexual selection of a trait or signal therefore arises from differences in the reproductive success of individuals related to differential expression of that trait (Andersson, 1994).

Sexual selection may take two forms. The first is intrasexual selection, involving traits that influence the outcome of competition among members of one sex for access to the other. Intrasexual selection typically operates on physical and behavioural features that play a role in the outcome of aggressive encounters between males over territorial disputes or access to females. The second form of sexual selection is intersexual selection. This leads to the evolution of secondary sexual traits or signals that determine the relative "attractiveness" of members of one sex to the other sex and are thus linked to mate choice. Mate choice can broadly be defined as "the tendency of members of one sex to mate non-randomly with respect to one or more varying traits in members of the other sex" (Heisler *et al.,* 1987). In many species, males attempt to attract females by exhibiting specific behaviours and investing in expression of sexual signals. These behaviours and signals may be highly variable among individuals but very specific in terms of when and how they are elicited. In many species, males produce these signals and mate choice is often observed in terms of female choice (Andersson, 1994).

Several mechanisms may be involved in the evolution of female choice for male secondary sexual signals or traits. These include:

- The acquisition of direct phenotypic benefits such as food, paternal care and access to resources of high quality. Females exhibiting a preference for a specific form of a male signal may obtain a direct benefit in terms of an increase in fecundity and a

reduction in their own reproductive costs (Price *et al.,* 1993). For direct benefits to drive the evolution of female choice there must be some obvious variation among males in terms of the benefits females can obtain, such as differences in paternal care. For example, males are likely to vary in their ability to protect and defend young as well as in their ability to provision for offspring. If a male sexual signal or trait reliably indicates the level of paternal care a male is likely to provide, and females are able to raise a larger number of healthy offspring by choosing a male possessing that form of a trait, selection will favour a mating bias for specific forms of that trait.

- Indictor traits that reliably reflect genetic quality (Grether, 2010). For a secondary sexual trait to be considered a reliable indicator of genetic quality it must fulfil four main criteria: females must show a preference for the trait, the trait must be costly to produce and maintain, the size of the trait must be correlated with a component of male fitness, and the component must have a genetic basis allowing it to be inherited by offspring (reviewed by Andersson, 1994). The evolution of female choice through indicator mechanisms is driven by indirect benefits that females obtain from a particular mating basis. For example, mating with an attractive male may result in the production of offspring that are of high genetic quality. The indicator traits hypothesis can broadly be split further into the handicap theory, the good genes hypothesis and the Hamilton-Zuk hypothesis.
    - The handicap theory attempts to understand how "honest" or reliable signals evolve and postulates that reliable signals must be costly, leading to condition-dependent signalling (Zahavi, 1975). Only individuals that can withstand the costs associated with the attractive expression of a particular trait are likely to be of high quality and should be preferred by females.
    - The good genes hypothesis states that females should select males whose traits signify a high level of genetic quality (Mays Jr and Hill, 2004; Kokko, 2001). In making this choice, females gain an indirect genetic advantage for their offspring. The increased quality or viability of offspring produced therefore negates some of the costs associated with being choosy.
    - The Hamilton-Zuk hypothesis postulates that sexual signals or ornaments are indictors of parasite and disease resistance (Hamilton and Zuk, 1982). This hypothesis assumes that females choose between potential mates based on secondary sexual traits and that the expression of these traits is limited by

parasitic infection or disease. Females should therefore choose males with the most elaborate or extreme variations of a particular trait as these individuals are likely to have a low parasite load or a high level of genetic resistance. As a result, this resistance will be inherited by offspring, increasing their survival rate and providing the indirect benefit of increased reproductive success to females.

- Exploitation of a pre-existing sensory bias not associated with mate choice (Ryan, 1990). Males may exploit a bias displayed by females to obtain a greater number of successful mating opportunities (reviewed by Endler and Basolo, 1998; Fuller *et al.,* 2005). This mechanism is thought to explain large difference in traits between closely related species as it produces a divergence in signalling systems that could lead to reproductive isolation. Initially males would not invest in traits to specifically attract females as it would not be beneficial for males to expend time and energy signalling if females are not choosy. A pre-existing sensory bias would provide the foundations for females to begin choosing between males and for variations in this male signal to become important. Additionally, when females respond incidentally to a male trait that correlates with a specific aspect of individual quality this may initiate a signal-preference co-evolution.
- Fisherian runaway selection, which refers to the genetic coupling between a male trait and the preference for that trait, eventually leading to self-reinforcing co-evolution (Fisher, 1915). The process of Fisherian runaway selection is initiated when a trait arises within a population via natural selection but is quickly favoured by sexual selection, even if it becomes extremely costly to maintain. Female choice for certain traits is not driven by the direct or indirect benefits gained as a result of a particular mating bias, but as a result of an arbitrary preference. For example, within a given population, genetic variation in the size of a male ornament such as a horn may exist. If males gain a fitness advantage as a direct result of possessing a particularly large ornament, large ornament size will be favoured by natural selection. If, within the same population, females are choosy there may also be genetic variation in female preference. As a result, females that arbitrarily prefer males with larger horns will produce sons with large horns and daughters that prefer males with large horns. Consequently, alleles for large horn size and alleles for a female preference for large horns will spread through the population at the same time. Over time males with large horns would begin to accrue increased

reproductive success owing to the female preference spreading through the population and a feedback "runaway process" develops (Fisher, 1930). As a result, Fisherian traits are more likely to lead to reduced genetic variability and female choice within a population than traits that are reliable signals of quality. This is because reliable indicators of quality allow females to accrue benefits as a result of mate choice that balance any costs associated with being choosy; selecting males based on Fisherian traits does not typically provide these benefits, thus, some of the costs of being choosy persist.

1.2 MALE SECONDARY SEXUAL SIGNALS

1.2.1 Visual signals

Sexually selected conspicuous colouring and exaggerated ornaments are displayed by males of many species. Male coloration, such as the orange and red plumage of many birds, is often reliant upon the presence of carotenoid pigments that cannot be synthesized naturally. As a result, these pigments must be obtained through the diet and are likely to be a direct indicator of a male's foraging ability or a reflection of the quality of resources within his territory (reviewed by Olsen and Owens, 1998). For example, male house finches (*Carpodacus mexicanus*) displaying the most intense and largest red plumage patches typically provide better paternal care, have increased offspring survival and thus enhance the reproductive success of females (Hill, 1991). Similarly, in the monogomous blue-footed booby (*Sula nebouxii*), the blue coloration of a male's feet is reduced when individuals are food deprived (Velando *et al.,* 2006); females respond to this change by altering reproductive investment and typically produce smaller and lighter eggs when the coloration of their mate is reduced (Velando *et al.,* 2006).

In addition to food availability and male condition, the strong correlation between carotenoid levels and immunological competency may be important for female choice (Chew, 1993). Individual males with compromised immune function typically exhibit duller coloration or a relatively small patch of pigmentation. Colouration could therefore provide females with a reliable signal of disease resistance. Addition of carotenoids to the diet of zebra finches (*Taeniopygia guttata*) increases beak coloration and improves immune system function (McGraw and Ardia, 2003). Similarly, male mallards

(*Anas platyrhynchos*) with the brightest beaks typically have higher immune responsiveness and improved sperm motility (Peters *et al.,* 2004), and male Lake Victoria cichlid fish (*Astatoreochromis alluaudi*) with the brightest beaks possess the largest territories and have the lowest parasite loads (Maan *et al.,* 2006). By choosing more brightly coloured males, females not only gain direct benefits such as access to higher quality resources, but they may also gain indirect benefits such as parasite resistance for their offspring. Colouration may also reflect immunocompetency due to the fact that the development of many sexually dimorphic signals is under the control of androgens, which may actually reduce immunocompetency (Folstad and Karter, 1992). Brighter or larger areas of pigment may therefore reflect the ability of a male to withstand costs such as reduced metabolic rate and partially suppressed immune function associated with high levels of testosterone required to maintain attractive colouration (Folstad and Karter, 1992). Despite limited support for this theory (reviewed by Roberts *et al.,* 2004), testosterone has been shown to be positively correlated with visual signals and negatively correlated with immunocompetence in several species (e.g. Verhulst *et al.,* 1999; Kortet *et al.,* 2003; Mougeot *et al.,* 2004; Kurtz *et al.,* 2007).

Anatomical structures and weapons can function as visual secondary sexual signals. Examples include elongated tails and exaggerated horns or antlers. The selection for long tails in males is particularly well studied in the widowbird family and in swallows where females typically prefer to mate with males that possess the longest tails (Andersson, 1982; Moller, 1990). In the red-collared widowbird (*Euplectes ardens*), tail length is positively correlated with male reproductive success and female choice targets exaggeration of tail length (Pryke *et al.,* 2001). Larger tails may impose higher energetic costs during development and impair general locomotion during foraging, territory maintenance and competition with other males. As a result, the size of a male's tail may be condition-dependent, reflecting not only male nutrition and viability, but also competitive ability and health status. For example, male barn swallows (*Hirundo rustica*) with longer tails have greater parasite resistance, higher paternity rates and sire longer lived offspring (Saino and Moller, 1993; Moller, 1994; Saino *et al.,* 1997). This suggests that tail length is a reliable predictor of male quality and that females should select mates based on the size of this anatomical feature.

1.2.2 Acoustic signals

Acoustic signals in the form of calls or songs are often some of the most easily perceived sexual signals. 'Song' in the context of a sexual signal can be defined as a 'long-range acoustic signal produced mainly during the breeding season' (Andersson, 1994); by contrast, calls are typically brief sounds comprising a simple acoustic structure. Songs and calls are often highly conspicuous and occur frequently during periods when females are actively selecting mates. For example, red deer (*Cervus elaphus*) stags have been known to call over 1000 times in a single 24 hour period during the rut (Clutton-Brock and Albon, 1979). Songs and calls may reflect male quality though specific characteristics such as amplitude and frequency, or via advertisement of territory ownership and competitive ability (reviewed by Searcy and Andersson, 1986). The rate of male calling or song production may be important in female choice as calls are likely to be energetically expensive to produce for long periods due to time spent away from foraging and high levels of physiological fitness that may be required. Call rate and the amount of time spent calling therefore has the capacity to accurately reflect male condition and access to certain nutritional and territorial resources (e.g. Hoback and Wagner Jr, 1997; Holzer *et al.,* 2003; Amorim *et al.,* 2010). For example, in red deer, females strongly prefer males that call at a high rate, a characteristic that accurately reflects fighting ability and reproductive success (McComb, 1991).

Specific call characteristics such as fundamental frequency, repertoire size and frequency range may also be important for female choice. For example, formants, which are the range of frequencies selectively amplified by the vocal tract, accurately reflect the size of a male's vocal cord length, which is typically related to body size (Fitch, 2000). Body size may be predictive of competitive ability, nutritional status, and access to high quality territorial resources, so females may prefer males that are able to produce a specific range of formants. For example, North American bison (*Bison bison*) bulls that produce lower frequency formants within their bellows achieve a higher mating success (Wyman *et al.,* 2012). Similarly, male fallow deer (*Dama dama*) that produce groans consisting of low formant frequencies are typically the most dominant and achieve the highest reproductive success (Vannoni and McElligott, 2008)

In species that utilise complex songs as sexual signals, song repertoires may vary in size or complexity between individuals, particularly in birds where vocal cues are the primary method of communication. Physiological and morphological constraints

associated with the production of songs may help to maintain honesty in this signal as the exertion and cognitive ability required to produce complex songs is likely to impose a high cost. For example, song repertoire size may be positively correlated with body size and condition (Doutrelant *et al.,* 2000; Kipper *et al.,* 2006), and cognitive ability and early developmental conditions that influence learning of a complex range of songs may impact the repertoire of individuals as adults (Nowicki *et al.,* 2000; Pfaff *et al.,* 2007). These potential costs and constraints may result in highly consistent reproduction of complex songs being difficult to achieve, leading to variability in song repertoire being under selection (Byers 2007; Botero *et al.,* 2009). Males that are highly skilled in song production may therefore be better equipped cognitively for behaviours such as foraging and paternal care. In support of this, song repertoire size is correlated with male reproductive success in several species (McGregor *et al.,* 1981; Hiebert *et al.,* 1989; Reid *et al.,* 2004).

1.2.3 Olfactory signals

Olfactory signals can be particularly important for communication between conspecifics, as unlike most visual or auditory signals, information can be obtained even when the signaller is absent. However, in contrast to auditory and visual signals that can travel in a more precise and directed manner, directionality and receiver identity is more difficult to control. In addition, the time taken for a signal to be detected can be problematic due to lack of speed in signal propagation (reviewed by Alberts, 1992). For olfactory communication to function effectively selection should therefore favour the evolution of signals that can be detected easily and effectively and produced at minimal cost. Semiochemicals facilitate communication between conspecifics and pheromones, a subclass of semiochemicals, are "substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific reaction, for instance a definite behaviour or developmental process" (Karlson and Luscher, 1959). Pheromones can be expelled from secretory glands such as the internal or external exocrine glands or excreted as components of urine or faeces (e.g. Jackson and Morgan, 1993; Kimoto *et al.,* 2005). Some pheromones are produced over the body from sebaceous glands in the hair follicles (reviewed by Smith and Thiboutot, 2008) and these glands are typically located in a position that allows the most efficient secretion and deposition of chemicals signals (e.g. around the face or on the back).

The dissemination of olfactory signals depends upon a range of factors including: the viscosity of the secretion, the location of the target substrate and the location of the receiver. Deposition via urination is particularly useful as it can be easily controlled and the location and pattern of these deposits can provide additional information. Depositing urine, faeces or glandular secretions in specific patterns and locations is referred to as scent marking, a behaviour that is often sexually dimorphic. Many theories regarding the functions of scent marking have been postulated (reviewed by Johnson, 1973). These include: labelling the local habitat for orientation and navigation, functioning as a deterrent, reducing the costs of aggressive interactions by allowing individuals to assess opponents from a distance, and acting as attractants to the opposite sex.

Scent marking is often physiologically costly and high investment may lead to reduced growth rate and small body size (Gosling *et al*., 2000). This cost is thought to help maintain honesty in olfactory signalling, as only high-quality individuals can afford to make extensive investments in these secondary sexual signals (Zahavi, 1977). Reliable information signalled by scent is therefore important to females as it can indicate a number of aspects of male quality such as genetic heterozygosity, health status and competitive ability. For example, females may be more likely to produce offspring with increased disease resistance and reduce the likelihood of contracting diseases themselves if infected males are avoided. As scent marks are often encountered before the scent owner, the presence of an honest indicator of health status allows infected males to be avoided. Females are typically less attracted to the scents of individuals carrying infections (Kavaliers *et al.,* 2004); in house mice (*Mus musculus*) the ability to distinguish between healthy and infected males is amplified when males are sexually stimulated or scent mark at a high rate (Zala *et al*., 2004). Females may discriminate between the marks of healthy and infected males and use this information to preferentially associate with uninfected individuals (Ehman and Scott, 2002). The frequency and pattern of scent marks also varies between individuals; dominant males typically scent mark at a higher rate than subordinate individuals (Ralls, 1971; Hurst, 1990), and dominance may be associated with the ability to defend and maintain a territory. As a result, females may prefer males that scent mark at the highest rate.

1.3 SEXUAL SIGNALLING IN MICE

In many species, males communicate information to potential mates using multiple sexual signals. Systems involving multiple signals or traits can be multicomponent, involving one sensory modality (e.g. visual signals) to convey information about different aspects of quality through separate signals, or multimodal, conveying information via different sensory modalities. Mice are particularly useful for investigating individual investment in multiple sexual signals as males produce sexual signals in two distinct modalities; auditory signals in the form of ultrasonic vocalisations and olfactory signals that include scent marks. In addition, laboratory mice are easy to manipulate and observe, and investigation of sexual signalling and courtship can be relatively straightforward as these behaviours are extremely well characterised (McGill, 1962; Portfors, 2007; Arakawa *et al.,* 2008). Finally, the limited genetic variation among individuals of each laboratory strain allows other factors that may influence behaviour and signalling to be explored while controlling for underlying genetic variation.

1.3.1 Auditory signals

Adult male mice produce ultrasonic vocalisations at a frequency of around 70 KHz when they encounter adult female conspecifics or female odours (Whitney and Nyby, 1979). The majority of male ultrasounds are emitted during the initial stages of an encounter with a female, when mutual sniffing and grooming are observed, and typically cease following mounting and ejaculation (Nyby, 1983). As a result, it has been suggested that ultrasound production serves a specific courtship function in mice (Nyby *et al*., 1977). Individual investment in this signal is also related to hormonal and social status; repeat episodes of social defeat or castration result in a short-term reduction in ultrasound production (Matochik *et al*., 1994; Lumley *et al*., 1999). Females are more likely to associate with a vocalising male over an individual that fails to emit any ultrasounds (Pomerantz *et al*., 1983). Thus, initial attraction and subsequent courtship may be mediated by this auditory signal. The syllables and specific call components produced by males differ between individuals and females prefer the calls of non-kin over those from familiar related individuals (Holy and Guo, 2005; Musolf *et al.,* 2010). This suggests the components of a male's call may function in individual or kin recognition, but little is known about the function of male calls in mate choice and

whether calling investment provides an honest signal of any specific aspects of male quality.

1.3.2 Olfactory signals

Olfaction is the primary modality through which many mammals detect, identify and communicate with conspecifics. Unlike the auditory signals that can be utilised for immediate communication when conspecifics are nearby, olfactory signals can persist in the environment for prolonged periods of time, providing valuable information to receivers even when the signaller is no longer present. As a result, scent signals allow communication with a high number of conspecifics over longer periods of time, and are important for a variety of behaviours including competition, territory defence, individual recognition and reproduction (Arakawa *et al.,* 2008; Hurst, 2009). Many scent signals are highly complex in composition and in the information they provide; to fully understand their importance in mediating behavioural responses of conspecifics it is essential to consider the many components of scent signals and the complex neural pathways that underlie detection and processing of this olfactory information.

Male mice invest in producing olfactory signals in the form of scent marks, which are attractive to females and typically encountered within the environment prior to an interaction with a male. The location and pattern of male marks allows individuals to advertise the size of their territory and signal their social dominance via countermarking the marks of intruders or subordinates (Rich and Hurst, 1998). By countermarking all other male scents, individuals are able to reinforce their dominance and ensure their scent remains the most recently deposited. This is important as the scents of other males may represent a challenge for dominance and create ambiguity for females when establishing the identity of the territory owner (Gosling, 1982), particularly as females are able to distinguish between two scent marks deposited in the same location based on the age of the scents and typically prefer the scent deposited most recently (Rich and Hurst, 1999). In addition, scent mark frequency is often indicative of social status; high quality dominant males scent mark at a higher rate than subordinate individuals (Desjardins *et al.,* 1973) and marking rate plays an important role in female preferences (Roberts and Gosling, 2003). In addition to scent mark pattern and rate, scent composition is important for communication with conspecifics as

specific components allow the life of a scent mark to be extended and provide males with an individual and genetic olfactory signature (Hurst *et al.*, 1998; Hurst *et al.,* 2001).

1.3.2.1 Olfactory signal components

One potential source of olfactory information in scent marks is MHC odour-type. The major histocompatibility complex (MHC) is a complex set of genes involved in self recognition in the immune system and influences the scents produced by individuals of many species (Singh *et al.,* 1987; Penn and Potts, 1998a; Strandh *et al.,* 2012). MHC peptides, which are typically between 4 and 20 amino acids in length, generally cannot pass through cellular membranes so are transported into the endoplasmic reticulum (ER) where they are presented to the immune system before being excreted into the extracellular space. Any cell within vertebrates, provided it has MHC immunity, has the ability to produce and excrete MHC peptide ligands. There are several hypotheses regarding how MHC type might act to mediate odour differences (reviewed by Penn and Potts, 1998; Penn 2002). These include:

1. The MHC molecule hypothesis – this theory suggests that MHC molecules themselves provide the odorants (Singh *et al.,* 1987). However, MHC based odour components appear to be volatile (i.e. when the large proteins present in mouse urine are removed, odours that differ in MHC-type remain distinguishable by other mice), and MHC proteins are large and involatile, suggesting that this hypothesis is unlikely.

2. The peptide hypothesis – MHC molecules alter the peptide metabolites in urine that provide the odour components (Singer *et al.,* 1997). This theory is consistent with evidence that odour differences due to MHC-type occur as a result of changes in the amount of specific urinary components that may potentially be metabolites of peptides.

3. The microflora hypothesis – MHC molecules may influence odour by influencing the populations of commensal microflora in urine (Schellinck and Brown, 1992).

4. The carrier hypothesis – MHC molecules carry volatile compounds that influence individual scent (Singh, 1999). The mixture and ratios of these

components carried by MHC molecules could provide a unique identity signature and degradation of the carrier molecule leads to release of these volatiles. This method is unlikely due to the hydrophilic peptide-binding properties of MHC molecules; conversion to hydrophobic aromatic-binding would be required for release of any volatile mixture of olfactory signal components.

Fragments of MHC protein complexes are found in many bodily secretions, and ligands released when MHC proteins are broken down may be filtered into urine and subsequently deposited in scent marks (Leinders-Zufall *et al.,* 2004). Evidence of MHC-based mating preferences has been established in a wide variety of species including rats, fish and humans (reviewed by Brown and Eklund, 1994; Jordan and Bruford, 1998; Penn, 2002), and in mice is thought to be based upon imprinting on odours during the rearing period in the nest (Penn and Potts, 1998b). However, evidence for MHC-based mate choice in mice remains inconclusive. For example, one study reported significantly fewer MHC-homozygous progeny in a semi-natural population of house mice than would be expected as a consequence of random mating (Potts *et al.,* 1991). Despite this, females did not show a pattern of settling in territories of MHC-dissimilar males. Alternatively, females may seek out dominant male territory holders that are relatively more MHC-disparate than the male who holds the territory they settle in; litters from females who mated with neighbouring territory holders contained 41% fewer homozygous offspring than if females had simply mated with the dominant male (Potts *et al.,* 1991). However, subjects used in this study were hybrid laboratory mice crossed with wild mice, which would have resulted in a substantial reduction in genetic variation among subjects compared to wild mice. Additionally, offspring were typed only for MHC, meaning parentage could not be determined and parental differences in relatedness or other genes that may be important for inbreeding avoidance could not be assessed. By contrast, freely breeding wild-derived mice in a separate study showed no avoidance of mates with the same MHC genotype (Sherborne *et al.,* 2007).

Although individuals of many species are thought to be able to determine the genetic identity of conspecifics based on MHC odour-type, the susceptibility of MHC peptide ligands to proteolytic attack may reduce their effectiveness as reliable signals of individual identity (Sherborne *et al.,* 2007). This is problematic for individual recognition, as signals of identity need to be highly stable. An example of an additional

olfactory component that may be used for genetic and individual recognition is the pattern of major urinary proteins (MUPs) produced by mice. Unlike the MHC complex, the only known function of MUPs is as a signalling component in urine and mice use the pattern of major urinary proteins for individual identification, regardless of MHC odour-type (Hurst *et al.,* 2001; Hurst *et al.,* 2005). As MUPs indicate individual identity and genetic heterozygosity, the pattern of MUPs within male scent is important to females when assessing the suitability of a male as a mate and avoiding extreme inbreeding (Sherborne *et al*., 2007). MUPs are encoded by a highly polymorphic complex of genes present on chromosome 4 and are expressed at very high concentrations in mouse urine (Mudge *et al.,* 2008); typically 99% of the protein present in mouse urine is made up of MUPs (Humphries *et al.,* 1999). Testosterone also plays a role in the hormonal control of differential expression of MUPs, such that some are male specific (Knopf *et al.,* 1983). Individual mice typically express between 8 and 12 different MUPs within their urine, each in different ratios depending upon the individual. This provides an opportunity for extremely high diversity in the MUP profiles of individuals even within small or relatively isolated populations (Beynon *et al.,* 2002).

MUPs are members of the lipocalin family and have a barrel structure that encloses a hydrophobic cavity capable of binding volatile ligands (Robertson *et al.,* 1998). MUPs bind several volatile male signalling pheromones including 2-sec-butyl 4,5 dihydrothiazole (thiazole) and 3,4 dehydro-exo-brevicomin (brevicomin), possibly concentrating and protecting these ligands from degradation (Novotny *et al.,* 1999). The tight binding of these ligands extends the life of a scent mark by increasing the amount of time pheromone release is effective, thus prolonging the length of time that a scent mark can convey information to conspecifics (Hurst *et al*., 1998).

One particular male-specific MUP, darcin, is particularly important for olfactory communication between conspecifics. Female sexual attraction to male urine is mediated by this male-specific pheromone and contact with darcin allows females to learn the associated volatile profile of a scent (Roberts *et al.,* 2010). This allows sexual attraction to remain inherent but selective towards particular males. Male odour also conditions a preference for its location, causing females to prefer to spend time in the location where a male odour has been encountered (Martinez-Ricos *et al.,* 2008); this induced spatial learning is mediated by darcin (Roberts *et al.,* 2012). Darcin is

responsible for binding most of the male-specific volatile, thiazole (Armstrong *et al.,* 2005), which is attractive to females and stimulates oestrus (Jemiolo, *et al.,* 1985; Jemiolo *et al.,* 1989). This tight binding extends the release of this pheromone over several hours after a scent is deposited. When the rate of release of this pheromone from male urine deposited on glass fibre discs is recorded, virtually all the thiazole in a scent mark dissipates within the first 24 hours (Armstrong *et al.*, 2005); however, females are still attracted to scent marks aged for 7 days (Roberts *et al.,* 2010), suggesting they are sensitive to incredibly low levels of thiazole or to other as yet unidentified components.

In addition to MHC, and MUPs, two sesquiterpenes, E,E-α-farnesene and E-β-farnesene, are produced in the preputial glands of male mice and added to urine when scent marks are deposited (Novotny *et al.,* 1990). These highly volatile compounds are attractive to females (Jemiolo *et al.,* 1991), stimulate oestrus (Ma *et al.,* 1999), and signal dominance in males (Novotny *et al.,* 1990). In contrast to the volatile components of mouse urine such as thiazole and brevicomin that are produced by all males, expression of farnesenes is partially suppressed in subordinates (Harvey *et al.,* 1989). The response of females to farnesenes in male urine also appears to require learning. Sexually experienced females prefer synthetic farnesenes over water in a simple two-choice test; sexually naive females only display this preference if farnesenes are presented at concentrations of between 50 and 100 times greater than that naturally excreted in male scent marks (Jemiolo *et al.,* 1991). This suggests farnesenes are not a primary attractant pheromone to virgin or inexperienced females, but that they acquire attractiveness through experience with conspecifics scents.

Other sources of chemical information include ESPs (exocrine-gland secreting peptides). Similar to MUPs, these peptides are encoded by a multi-gene family, but these genes are present on chromosome 17 rather than chromosome 4 (Kimoto *et al.,* 2007). These proteins range in size from 5 to 15kDa and are typically expressed in the facial extraorbital lachrimal, Harderian and submaxillary glands. Some ESPs are sex or laboratory strain specific but the extent to which expression of these proteins varies between individuals is not yet known (Kimoto *et al.,* 2007). The ESP family consists of around 38 different proteins in mice, roughly 10 proteins in rats and is absent from humans. This suggests that rapid molecular evolution in response to species specific signalling requirements and constraints may have occurred. One particular ESP, ESP1, is only expressed in the tears of males and stimulates a specific subset of receptors (V2)

in neurons of the accessory olfactory system (Kimoto *et al.,* 2005). This suggests a role for this peptide as a pheromone, providing information about the sex, strain and species of the individual. In addition to male-specific ESP1, ESP36 is expressed solely in the female extraorbital lacrimal gland (Kimoto *et al.,* 2007), and ESP 22, secreted in the tears of 2- to 3-week-old mice, acts as a pheromone to inhibit sexual behaviour of adults towards juveniles (Ferrero *et al.,* 2013).

1.3.2.2 Processing scent signals - the olfactory system

Olfactory signals that are important for sexual communication in mice comprise complex mixtures of both volatile and involatile components. The main olfactory system (MOS) detects and processes airborne scent components such as volatile chemicals and small peptides. By contrast, the complimentary accessory olfactory system (AOS) is able to detect large, involatile molecules when an animal makes direct contact with a scent. Recognition and assessment of conspecific olfactory signals has been shown to involve important interactions between the main and accessory olfactory systems, which often control sexual attraction, mate recognition and sexual behaviour through overlapping processing (Keller *et al.,* 2009). For example, when direct contact with a scent is possible, female mice show a consistent attraction to spend more time near male scent than female scent, regardless of whether the scent or scent owner has been encountered previously. By contrast, when contact with the scent is not possible, females only show an attraction to the airborne components of male scent from individuals whose scent they have previously contacted (Ramm *et al.,* 2008). This system is essential in allowing individuals to detect the presence of scents in the environment from a distance; subsequently, an animal may be stimulated to approach a scent to gain further information, particularly if the scent has not been contacted before. Females can therefore gain essential information regarding the suitability, attractiveness and quality of potential mates through direct contact with a scent. To assess how specific scent components influence the behavioural response of females to male scent marks in the environment, it is therefore essential to consider the physiology of the olfactory system and how it processes these different components of important olfactory signals.

The organisation and structure of the olfactory system across different species is highly variable. However, all olfactory systems share a common trait: the presence of

olfactory sensory neurons (OSNs). These neurons are often clustered together in special receptor organs and the basic pathways for processing of odours or chemical signals are initiated by the binding of odorant molecules to receptors in the cell membranes of OSNs. Olfactory systems typically detect and discriminate a vast number of different odours, made possible by the large range of receptor types and connections between individual neurons (Malnic *et al.,* 1999; Buck, 2004). Most vertebrates, including mice, possess a dual olfactory system comprising main and accessory systems that can detect odours and pheromones individually or through combined and overlapping processing (reviewed by Kelliher, 2007).

In the main olfactory system, the main olfactory epithelium (MOE) is connected to the main olfactory bulb (MOB). The MOE contains approximately two million olfactory sensory neurons, interacts directly with inhaled odours and is finely tuned to detect a broad range of molecules (reviewed by Mori *et al.,* 1999). The MOE contains OSNs and basal cells, which serve as olfactory stem cells and have short life spans of no more than 60 days (Chen *et al.,* 2004). OSNs are covered by cilia, which protrude into the mucus lining of the epithelium and act as the first point of contact between odour molecules and target receptors (Strotmann *et al.,* 2004). Each OSN expresses one of either the 1000 types of G protein-coupled seven-transmembrane odourant receptor (Buck and Axel, 1991; Malnic *et al.,* 1999), 15 trace amine-associated receptors (TAARs) or receptor guanylyl cyclase D (GC-D) (Young *et al.,* 2007; Nei *et al.,* 2008.). Odourant receptors in the mouse have been classified into two distinct groups, class I and class II, based on amino acid sequence homology (Zhang *et al.,* 2004). These receptors can be further divided into subfamilies on the basis of genetic sequence similarities; recent evidence suggests members of each of the more than 200 subfamilies identified recognise the same type of odour molecule structure, but that each individual member recognises different variants of that structure (Malnic *et al.,* 1999; Godfrey *et al.*, 2004). This characteristic is likely to be important in discrimination of very closely related odours or those with similar structures. Individual odour molecules can therefore be detected by a broad range of receptors; conversely, each receptor protein is broadly tuned to detect a range of odours (Buck, 2004). Whilst odourant receptors exhibit broad receptive ranges, other receptor types appear to be tuned to more distinct molecular features of odour molecules. For example, TAARs function as receptors for volatile amines and GC-D neurons recognise specific peptides and CO_2 (reviewed by Ihara *et al.,* 2013).

OSNs are connected to the main olfactory bulb via single axons and all neurons expressing a particular receptor protein converge onto mutually exclusive glomeruli (spherical structures where OSN axons terminate) within a specific region of the MOB. This projection pattern is defined by two principles; "zone-to-zone projection" and "glomerular convergence" (reviewed by Mori *et al.,* 1999; **Figure 1.1a**). The "zone-to-zone projection" principle is based on the clustering of receptor proteins in four distinct groups according to their spatial expression within the MOE. The groups of receptor protein expression are known as zones I, II, III and IV and are arranged in a pattern from the dorsomedial region of the epithelium (zone I) to the ventrolateral region (zone IV) (Ressler *et al.,* 1993). Within each zone, there is no distinct organisation of receptor expression, thus, neurons expressing different receptors intermingle. Despite this, receptor proteins with highly homologous amino acid sequences tend to be appear within the same local area (Malnic *et al.,* 1999). This organisational layout is also present within the MOB where glomeruli are organised into distinct zones (Schwob and Gottlieb, 1986). "Glomerular convergence" refers to each of the 1800 glomeruli within the rodent MOB acting as a centre for the convergence of several thousand neurons that each expresses the same, single receptor protein (Royet *et al.,* 1998). Additionally, the neurons present in zone I of the MOE project to glomeruli within zone I of the MOB (Vasser et al., 1994); odorant information detected within a specific zone of the MOE is therefore transmitted to glomeruli within a corresponding zone of the MOB.

Although individual OSNs within the olfactory system project to a single glomerulus within the MOB, these hubs of innervation are not isolated. Each glomerulus interacts with others via local interneurons and periglomerular cells (Mori *et al.,* 1999); mitral and tufted cells also make connections directly with each other as they carry signals from the glomeruli to the processing centres in the brain (Rall and Shepherd, 1968). The interconnectivity of these cells provides the network with feedback inhibition, and more importantly, lateral inhibition of neighbouring mitral or tufted cells (Isaacson and Strowbridge, 1998). Connections between glomeruli allow mitral and tufted cells of one glomerulus to potentially inhibit the cells of a neighbouring glomerulus, increasing the likelihood that a single odour can be distinguished from a complex mixture of similar molecules (Yokoi *et al.,* 1995). This ability to regulate signal transduction plays a central role in processing of olfactory information.

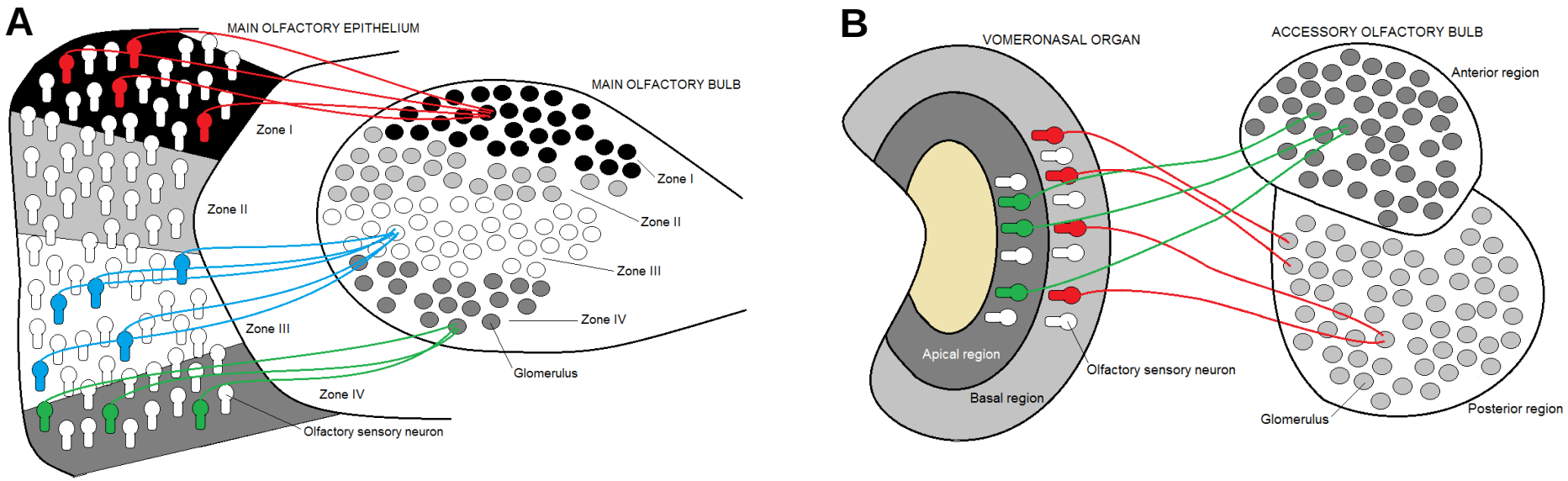


Figure 1.1 Olfactory system projections from sensory organs to processing centres.
Schematic of (A) axonal projection from the main olfactory epithelium to the glomeruli in the main olfactory bulb and (B) axonal projections between the vomeronasal organ and accessory olfactory bulb glomeruli (Adapted from Mori *et al.,* 1999).

In mice, as in most mammals, the main olfactory system is complemented by an accessory olfactory system. This dual processing provides many benefits including an increase in the number and type of odours that can be detected. The accessory system typically consists of the vomeronasal organ (VNO), or Jacobson's organ, that projects to the accessory olfactory bulb (AOB) and plays a role in detection of pheromones (Dulac and Torello, 2003; Halpern and Martinez-Marcos, 2003). The VNO is located in the base of the nasal cavity, is often tubular in shape and has a crescent-shaped lumen filled with fluid that protects the receptor neurons (Doving and Trotier, 1998). Uniquely, VNO neurons are isolated from the air that may pass through the nasal cavity during normal respiration. As a result, specific activation of a vascular pump is required for the VNO to function (Meredith, 1994); in many species the flehmen response, or pronounced lip-curl and closure of the nostrils, is associated with stimulation of the VNO (reviewed by Estes, 1972).

In mice as in many species, the VNO sensory epithelium is the first point of contact for odorant molecules in the accessory olfactory system. In contrast to receptor neurons in the MOE that possess cilia, the neurons within the VNO have apical microvilli on their surface. In addition, unlike the variety of receptors found in the MOE, each VNO neuron expresses either the formyl peptide receptor (FPR) or one of two G-protein linked receptor types, V1 and V2, each found in a distinct region of the VNO (Dulac and Torello, 2003; Liberles *et al.,* 2009). As in the main olfactory system there is "zone-to-zone" projection between the MOE and the glomerular layer of the AOB. Sensory neurons found in the apical regions of the VNO express V1 receptor proteins and project to the anterior region of the AOB; neurons in the basal region of the VNO express V2 receptor proteins and project to the glomeruli in the posterior region of the AOB (Halpern *et al.,* 1998; **Figure 1.1b**). The activation and physiological response of VNO neurons is different to neurons of the MOE that fire single bursts of action potentials and require pulsatile activation to continue firing (reviewed by Keverne, 1999); by contrast, VNO neurons maintain a constant current showing slow adaptation after firing. Although an increase in firing rate occurs as the strength of the stimulus increases, weak stimuli are able to activate VNO neurons if stimulation is persistent (Liman, 1996). These features may explain the need for prolonged exposure to some pheromones for the induction of changes to reproductive physiology and behaviour.

Despite expressing relatively few receptor types, the sensory neurons of the VNO are able to discriminate and process a high variety of odorant molecules and pheromonal peptides. V1 receptors recognise small, volatile pheromones and steroids, while V2 receptors and FPRs detect small peptides (reviewed by Ihara *et al.,* 2013). In signalling individuality, members of a species may share many similar odour components but in different proportions, requiring increased sensitivity for these differences to be reliably detected. In the MOB, each olfactory neuron carrying a specific receptor type projects to a single glomerulus, maximising sensitivity to a wide variety of odorant molecules. By contrast, the connections in the AOB are much more variable; neurons expressing one receptor type project to many different glomeruli, and each of these glomeruli receive input from neurons expressing all types of receptors (Keverne, 1999; Rodriguez *et al.,* 1999). These differences in connectivity may reflect the different functions of the main and accessory olfactory systems. Whilst the main olfactory system processes a wide variety of odours, often at low concentrations, the accessory olfactory system processes more specific olfactory cues that may contain mixtures of components that are similar, but not identical, between individuals (Sorensen, 1996). In addition, the accessory olfactory system is more important for social behaviour in naïve animals than in experienced individuals. As a result, having numerous, interconnected glomeruli may allow for greater plasticity and remodelling of existing circuits as an individual matures.

Although the main and accessory olfactory systems comprise different receptor proteins and have different projections to processing centres in the brain, they are unlikely to always operate independently. Sensory neurons in both the VNO and MOE respond to some specific MHC peptides and the MOE can gain access to involatile olfactory cues following direct contact with a scent (Spehr *et al.,* 2006). Additionally, the response of VNO receptors to MHC peptide ligands is not unique (Leinders-Zufall *et al.,* 2004); neurons in the MOE express receptor proteins that can also detect peptide ligands similar to those that form part of the MHC complex (Spehr *et al.,* 2006). In support of this, mice with a disrupted VNO are still able to discriminate odours based on MHC-type (Wysocki *et al.,* 2004).

Despite the ability of the main olfactory system to respond to some involatile odour components, removal or disruption of the VNO in rodents impairs normal responses to conspecific pheromones and disrupts sexual behaviour. This effect is more

prominent in sexually naïve individuals; little disruption is observed if VNO function is impaired in sexually experienced adults (reviewed by Keller *et al.,* 2009). Brain mapping studies show sexually experienced animals are able to utilise input from the MOS to partially replace input from the accessory olfactory system if the VNO is disrupted (Fewell and Maredith, 2002). This suggests the VNO plays a role in initial detection and learning of odours and that the MOS is subsequently capable of functioning more independently in experienced individuals. For example, MUPs provide fixed information regarding sex and identity, which, due to their involatile nature, are detected through the VNO (Leinders-Zufall *et al.,* 2000). Following initial attraction to a scent by the airborne volatiles, learning of an individual's scent signature requires direct contact with the involatile components (Ramm *et al.,* 2008). This combined processing in the main and accessory olfactory systems allows females to subsequently identify individuals on the basis of the associated volatiles alone. As the volatile components are more readily detected at a distance, time and energy expenditure could be reduced if females can distinguish familiar from unfamiliar males based on these volatile signals; if unfamiliar, the female could contact the mark to obtain further information.

Other important olfactory organs include the septal organ and the Grüneberg ganglion. The septal organ (SO) is covered with a chemosensory epithelium and is located at the ventral base of the nasal septum in many mammals including mice. It is separate from the main olfactory epithelium but contains similar sensory neurons that are ciliated and project to the MOB. Within the SO a few thousand neurons are organised into three layers of cells and each neuron has a similar morphology, receptor protein expression and projection pathway to neurons in the MOS (Weiler and Farbman, 2003; Kaluza *et al.,* 2004). Most of the receptors in this organ are expressed by only a few of the sensory neurons, but receptor types OR244-3 and OR256-3 are expressed by a uniquely high proportion (Kaluza *et al.,* 2004; Tian and Ma, 2004). This suggests they play a significant role in the function of this organ; by concentrating function to a few broadly tuned olfactory receptors the SO may serve as an effective general odour detector, acting as a secondary organ of the MOS. In contrast to the neurons in the MOE or VNO, there is no distinct spatial distribution within the SO or in the axonal projections to the olfactory bulb. Despite this, all nerve fibres from the SO pass across the MOE and make connections with glomeruli in the MOB, including a subset of specific septal glomeruli that receive input exclusively from the SO (Levai and Strotmann, 2003). As septal neurons project to some of the same glomeruli as

neurons from the MOE, this organ may be important for monitoring general airflow during respiration and promoting targeted sniffing when required. The presence of distinct septal glomeruli also suggests this organ may serve a specific function that has not yet been identified (reviewed by Breer and Strotmann, 2005).

Another potentially important component of the olfactory system is the Grüneberg ganglion (GG) (Grüneberg, 1973). Unlike the neurons in the SO, Grüneberg neurons are morphologically distinct from those in the VNO or MOE. These neurons project to specific 'necklace glomeruli' located in the most caudoventral and caudodorsal regions of the MOB (Koos and Fraser, 2005). Cells in the GG do not express prominent cilia and the absence of these structures means detection of odours may not be via the airflow in the nasal cavity but via particles that are able to penetrate the thin mucosa. Additionally, the position of the organ at the very tip of the nose would allow direct contact and uptake of involatile molecules meaning the GG could be important in detecting involatile scent components (Fuss *et al.,* 2005); the region around the GG contains several glands that produce secretions that may assist in the transport of odourant molecules to neurons. Unlike the MOE and VNO, which continue development long after birth, this olfactory organ is mature at birth, but during adulthood the number of cells in the GG decreases (Grüneberg, 1973), suggesting it plays a more prominent role in neonatal and pre-pubertal animals. Evidence from recent studies suggests this olfactory subsystem may also play an important role in the detection of alarm pheromones (Brechbühl *et al.,* 2008), which signal injury, distress or the presence of predators to conspecifics. Additionally, GG neurons respond to immediate declines in temperature within a given range and may function as finely tuned cold detectors (Schmid *et al.,* 2010). This suggests this olfactory organ may play an important role in the processing of, and subsequent behavioural response to, olfactory cues associated with threatening, aversive or stressful situations.

1.3.2.3 Higher order processing of scent signals

To establish whether the neural pathways or neurophysiology of specific regions of the brain are influenced by detection and processing of olfactory signals, the areas connected to the olfactory system, either indirectly or directly, must be identified. In addition, identifying these important processing centres and the connections between

them is important when considering how behaviours such as individual recognition, mate searching and mate choice may be mediated by olfactory signals present in the environment. Axons of the output neurons from the olfactory bulbs project to higher order processing centres in several different regions of the brain (Shipley and Adamek, 1984; **Figure 1.2; Table 1.1**). Ultimately, connections are made with the medial dorsal nucleus of the thalamus, which in turn projects to the orbitofrontal cortex, a region involved in conscious perception of smell (reviewed by Shepherd, 2007). The hippocampus, an area of the brain important for memory formation, learning, and the processing of spatial information (Jarrard, 1995), is also connected to the olfactory system via several intermediate processing centres. In most species, the entorhinal cortex (EC), which is involved in odour processing, is one of the main sources of hippocampal input (Tamamaki and Nojyo, 1995), establishing a link that may be important for learning and storing paired odour and spatial information. Within the hippocampus the flow of information occurs largely in one direction as signals are propagated through a series of tightly packed cell layers starting with the dentate gyrus, where neurogenesis occurs, and proceeding to the CA3 and CA1 layers (Yeckel and Berger, 2001). Each of these layers contains extensively connected circuitry and the hippocampus itself sends out a vast number of projections to other areas of the brain. The hippocampus is also involved in the formation of memories relating to previous experiences allowing individuals to detect novel stimuli and use previous experience to guide an appropriate response (reviewed by Tulving and Markowitsch, 1998).

Impairment of hippocampal function typically results in an inability to correctly utilise contextual and spatial information whilst performance in tasks that involve learning without spatial information is generally unchanged (reviewed by Jarrard, 1995; Broadbent *et al.,* 2004). However, the hippocampus has been shown to be required for social recognition in rodents (Maaswinkel *et al.,* 1996; Uekita and Okanoya, 2011), a behaviour typically mediated by olfactory signals, suggesting this region of the brain is not only important for processing and storing spatial information, but that processing of some socially relevant odours relies on hippocampal function. The ability to process olfactory and spatial information simultaneously would be advantageous, particularly in the context of mate choice when females must locate and assess the scent marked territories of multiple males. Forming an association between a preferred scent and the location of that scent would allow females to be selective and return to the territory of a preferred male when ready to mate.

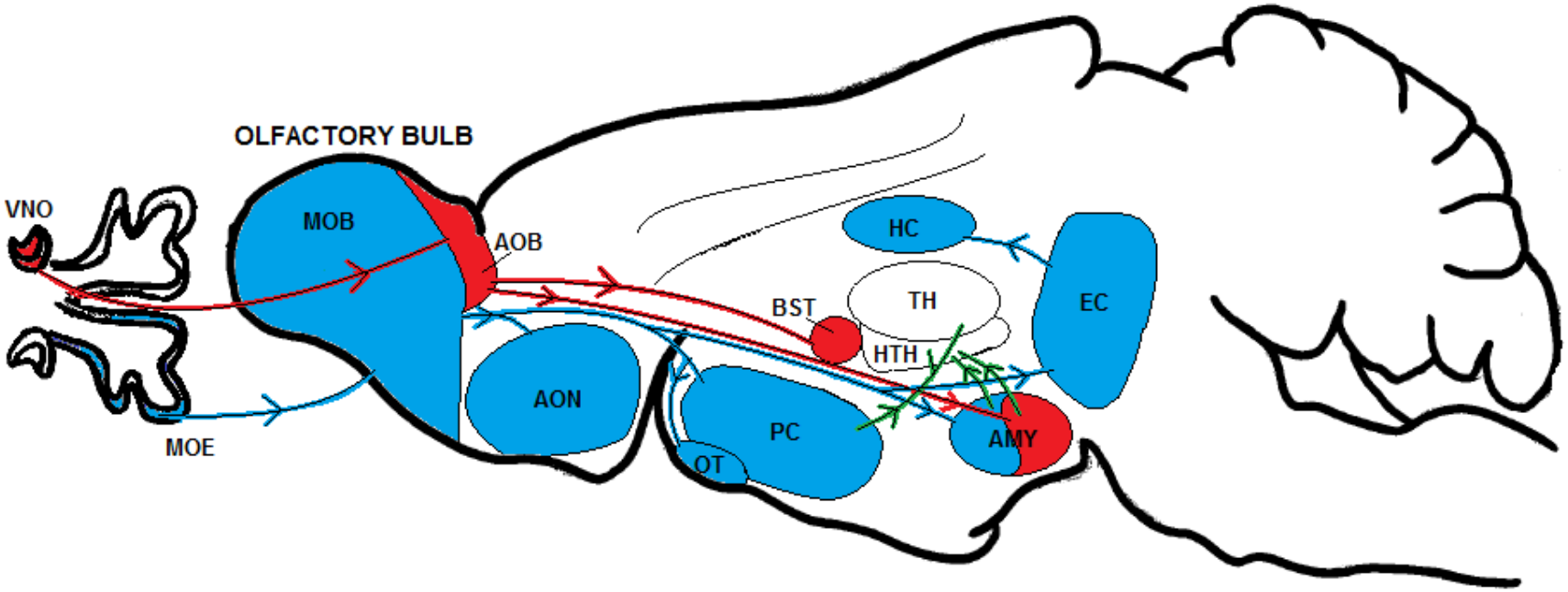


Figure 1.2 Schematic of brain regions important for mouse olfactory processing

Main olfactory pathway is shown in blue; accessory system pathway is shown in red. Abbreviations: MOE, main olfactory epithelium; VNO, vomeronasal organ; MOB, main olfactory bulb; AOB, accessory olfactory bulb, AON, anterior olfactory nucleus; BST, bed nucleus of stria terminalis; OT, olfactory tubercle; PC, piriform cortex; EC, entorhinal cortex; AMY, amygdala; HTH, hypothalamus; TH, thalamus; HC, hippocampus. (After Canavan *et al.,* 2011.)

Brain Region	Function in Olfactory Processing
Anterior Olfactory Nucleus - AON	The AON is located behind the olfactory bulb and in front of the piriform cortex (PC). It consists of two structures: a thick ring of cells encircling the end of the olfactory penduncle (pars externa) and the large pars principalis. Axons of mitral cells project from the MOB and pass through the AON before connecting to the piriform cortex (Ferrer, 2004). Some axons make synaptic connections at the AON, but the AON plays a role in distributing information to the PC as well as other processing areas in the cortex. Its primary function is therefore to regulate information flow between the regions of the brain that are important in odour processing (reviewed by Brunjes *et al.,* 2005).
Olfactory Tubercle - OT	The OT is a processing area of the olfactory cortex that plays a role in reward behaviours (reviewed by Ikemoto, 2007). It receives direct input from the MOB and contains tightly packed cell clusters known as the islands of Calleja (reviewed by Fallon *et al*., 2004). Although connected to the olfactory system, its specific role in odour processing has not yet been identified.
Piriform Cortex – PC	The piriform cortex (PC) is a key output target of the MOB. Mitral cell axons project to multiple regions in the PC and odours evoke widely distributed PC activity (Ojima *et al.,* 1984; Illig and Haberly, 2003). Different odours activate unique sub-populations of neurons distributed across the PC without spatial preference and projections of individual glomeruli are broadly distributed throughout the entire PC (Stettler and Axel, 2009; Ghosh et al., 2011; Sosulski et al., 2011).
Cortical Nucleus of Amygdala – CN	The CN is the smallest portion of the amygdaloid complex. It is known as the olfactory amygdala as the primary input to this area is from the olfactory bulb and cortex (Kevetter and Winans, 1981). Following learning, long-term memory is not formed instantaneously, rather, information is slowly assimilated into long-term storage (memory consolidation) (reviewed by Dudai *et al*., 2004). While the amygdala is not itself a long-term memory storage site and learning can occur without it, one of its roles is to regulate this memory consolidation (McGaugh, 2004). It also makes connections with the hippocampus.
Entorhinal Cortex - EC	The EC is located in the medial temporal lobe and functions in memory and navigation. The EC is the main interface between the hippocampus and neocortex and plays an important role in spatial memory formation and consolidation (Steffenach *et al.,* 2005). The superficial layers receive highly-processed input from every sensory modality including the olfactory system and subsequently project to the dentate gyrus in the hippocampus (reviewed by Heinemann *et al.,* 2006).
Bed Nucleus of Stria Terminalis - BNST	The stria terminalis marks a line of separation between the thalamus and the caudate nucleus. It carries fibres from the amygdala to the septal nuclei, hypothalamic, and thalamic areas of the brain. It also carries fibres projecting from these areas back to the amygdala (Weller and Smith, 1982). The activity of the bed nucleus correlates with anxiety and it is thought to act as a relay site within the hypothalamic-pituitary-adrenal axis; its activity is regulated in response to acute stress (reviewed by Walker *et al*., 2003).

Ventro-medial Hypothalamic Nucleus	The ventromedial nucleus is present in the hypothalamus and is involved in feeding, fear, thermoregulation, play behaviour and sexual activity (Colpaert, 1975; Kow *et al.,* 1992). This region has a role in male vocalisation, scent marking and in female sexual behaviours such as lordosis (Pfaff and Sakuma, 1979; Harding and Mcginnis, 2005).
Nucleus Accumbens (Acb)	The nucleus accumbens is a collection of neurons and forms the main part of the ventral striatum. It plays an important role in reward, pleasure, laughter, addiction, aggression and fear (Ikemoto and Panksepp, 1999; Levita *et al.,* 2002). The output neurons of the Acb project to the ventral pallidum (VP), which in turn projects to the medial dorsal nucleus of the thalamus and prefrontal cortex. Major inputs to the Acb come from dopaminergic neurons located in the ventral tegmental area (VTA) and from the CA1 and ventral subiculum of the hippocampus (Floresco *et al.*, 2001).
Ventral Tegmental Area (VTA)	The ventral tegmental area (VTA) is a group of neurons located close to the midline on the floor of the midbrain. The VTA is the germinal centre of dopaminergic cell bodies and is important in the reward circuitry of the brain (Floresco *et al.,* 2001). It processes various types of emotion output from the amygdala, playing a role in avoidance and fear-conditioning (Mahmoodi *et al.,* 2011). VTA neurons respond to novel stimuli, unexpected rewards, and reward predictive sensory cues (Takahashi *et al.,* 2009).
Medial Preoptic Area (MPA)	The medial preoptic nucleus is sexually dimorphic, releases GnRH and controls copulation in males and maternal behaviour in females (Rosenblatt and Komisaruk, 1977; Gorski *et al.*, 1978; Arendash and Gorski, 1983).

Table 1.1 Brain regions important for olfactory processing.
This table contains a brief outline of the functions of specific regions important for processing olfactory information. Input and output connections with the olfactory bulb and other areas are identified.

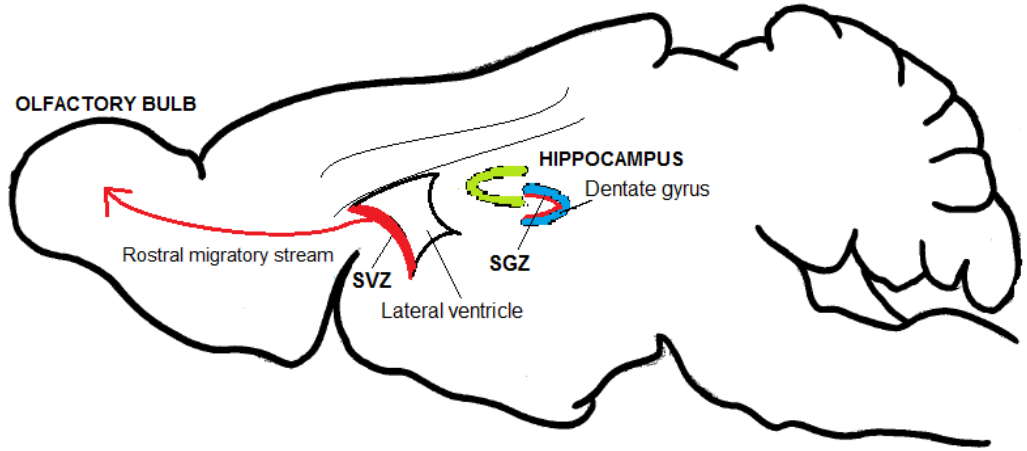
1.4 ADULT NEUROGENESIS

For many social mammals, the brain faces an important challenge. In many species, communication between conspecifics for kin recognition, territory defence and mate choice is mediated by olfactory signals containing components that signal both fixed and variable information that the brain must process simultaneously (reviewed by Eisenberg and Kleiman, 1972). For example, the innate response to fixed species and sex information requires neurological circuitry to be maintained. Conversely, the response to factors that are variable such as competitive ability and health status must remain plastic, implying that underlying circuits must also be flexible and able to adapt. Additionally, the brain must constantly adjust to the growth of an individual and its environmental experiences during early development. Ultimately, the adult brain must maintain behaviour and preserve the underlying neural networks whilst allowing other important circuits to remain plastic and adaptable.

Neurogenesis, or the generation and integration of new neurons, persists throughout life in two structures of the adult mammalian brain: the olfactory bulb and the hippocampus (**Figure 1.3**). Neurogenesis is thought to be involved in the formation of olfactory and spatial memories and has been suggested to be a potential mediator of the essential balance between circuit stability and plasticity (reviewed by Lledo *et al.,* 2006). Neurogenesis is a complex process that begins with the division of precursor cells and ends with the integration of new fully functioning neurons. In addition, cells must migrate following specified routes to their final regions of integration and form synaptic connections within the existing network without disrupting current function. Not all cells generated during the early stages of proliferation survive to maturity but undergo intense selection; as many as 60% of newly generated cells die before reaching maturity (Dayer *et al.,* 2003). This process of development and selection is influenced by a wide variety of factors including hormones, social experience and changing environmental conditions, ultimately regulating new neuron growth that is dependent upon the changing neurological needs of an individual (reviewed by Abrous *et al.,* 2005).

1.4.1 Olfactory system neurogenesis

In the brains of adult mammals, new neurons are added to the olfactory bulb throughout life. These cells are generated in the subventricular zone (SVZ), a cellular



STAGE OF DEVELOPMENT	Proliferation	Migration	Migration	Differentiation	Proliferation	Migration	Migration	Differentiation
CELL TYPE	B	C	A	Neuron	1	2a	2b	3 and Neuron
CELL IDENTITY	Astrocyte and putative stem cell	Transit amplifying progenitor	Migrating neuroblast	Immature and mature neurons	Putative stem cell	Transit amplifying progenitor	Immature neuron	Mature neuron
IDENTIFIED MARKERS	GFAP Nestin Ki67	Dlx2 Nestin	PSA-NCAM Dlx2 Tuj1	DCX NeuN Calbindin	GFAP Ki67 PCNA	Nestin	DCX Calretinin	NeuN Calbindin

Figure 1.3 Neurogenesis in the adult brain.
Neurons generated in the dentate gyrus remain in this region, being incorporated into hippocampal circuits. By contrast, neurons generated in the lateral ventricles of the forebrain migrate via the rostral migratory stream to the olfactory bulb. The table indicates the sequence of developmental steps, cell types and histological markers that can be used to identify cells at each step of olfactory (*light grey*) and hippocampal (*dark grey*) neurogenesis.

layer found within the walls of the forebrain lateral ventricles (Luskin, 1993; Lledo and Saghatelyan, 2005; **Figure 1.3**). This region contains a large population of neural stem cells within a rostrocaudal gradient of proliferative activity that is correlated with the ability of cells to divide and produce early lineage neuronal precursors (Lois and Alvarez-Buylla, 1994; Seaberg and van der Kooy, 2002). In mice, the SVZ is located several millimetres from the olfactory bulb (Lois and Alvarez-Buylla, 1994) and cells must traverse a complex network of interconnected pathways before being organised into chains and beginning migration to the olfactory bulb via the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). The mechanics behind cellular migration in the RMS are still poorly understood, but the close association between the migrating chains of neuroblasts and the blood vessels within the RMS suggest these vessels may provide anchorage (Whitman *et al,* 2009).

Within the SVZ there are at least four different cells types, each displaying unique morphological and molecular properties and representing a different step in neurogenesis (Doetsch *et al.,* 1997). These cells are named type- A, B, C and E:

1. Type-A cells – these are young migrating neuroblasts that form chains and are sheathed by astrocytes, or type-B cells. Type-A cells exhibit an elongated morphology during migration and express proteins such as PSA-NCAM as they move in a rostral direction towards the olfactory bulb (Wichterle *et al.,* 1997). Migration can also occur in other directions and some of the developing neuroblasts move tangentially through the RMS or even stop and reverse direction (Bolteus and Bordey, 2004; Lois and Alvarez-Buylla, 1994).
2. Type-B cells – these cells form a protective sheath around type-A cells and interact closely with type-E cells. They express nestin and GFAP, have short cilia and display astrocytic properties (Doetsch *et al.,* 1999b). Type-B cells are the progenitors of all active neurogenic cell types within the SVZ and generate transient amplifying type-C cells that subsequently generate type-A cells (B → C → A) (Doetsch *et al.,* 1999a).
3. Type-C cells – these cells are spherical, highly proliferative progenitor cells that form clusters next to chains of type A-cells. They are known as transient amplifying progenitors and express proteins such as nestin.
4. Type-E cells – these are the only cells in the SVZ that are not actively involved in neurogenesis. They are ependymal ciliated cells and face the lumen of the ventricles

to aid in the circulation of cerebrospinal fluid around the brain. Initially it was suggested that even type-E cells within this region were proliferative (Johansson *et al.,* 1999), but the progeny of these cells do not have the ability to self-renew or generate neurons (Chiasson *et al.,* 1999). Type-E cells therefore play a role in separating the active region of the SVZ from the ventricle cavity.

Despite the presence of all these cell types throughout the SVZ of the lateral ventricles, this region is not homogenous. Precursor cells generated within different areas give rise to different subtypes of neurons within the olfactory bulb (Merkle *et al.,* 2007). For example, periglomerular cells that synapse within and between the glomeruli of the olfactory bulb are generated from stem cells in the anterior-medial areas of the SVZ. By contrast, deep granule cells are generated in the ventral region of the SVZ. Further, all stem cells retain an inherent potential that cannot be altered and transplantation of precursors from one area of the SVZ to another does not alter their fate (Merkle *et al.,* 2007). During the early stages of neurogenesis, migrating cells have membrane properties similar to immature neurons and do not possess any synaptic connections, despite being able to fire action potentials (Carleton *et al,* 2003). However, once maturation begins the cells develop spontaneous inhibitory and excitatory synaptic currents; by day 30 the new cells are indistinguishable from mature granule cells already present (Petreanu and Alvarez-Buylla, 2002).

1.4.2 Hippocampal neurogenesis

In contrast to olfactory neurogenesis, the germinal zone for hippocampal neurons is not located near the walls of the lateral ventricles. Instead, the germinal subgranular zone (SGZ) is located in the interface between the granule cell layer (GCL) and the hilus of the dentate gyrus (DG), deep within the hippocampus. Initial studies suggested progenitor cells were lineage-restricted and had limited potential for self-renewal (Bull and Bartlet, 2005), but the use of advanced techniques recently identified the multipotent multi-lineage potential of the precursor cells within the DG of rodents (Babu *et al*, 2007). The process of neurogenesis within the DG comprises several developmental stages (Kempermann *et al*, 2004). At each stage distinct cellular proteins are expressed and the developing cells exhibit specific morphologies and patterns of activity. As development progresses the proliferative activity of cells decreases in

conjunction with an increase in differentiation towards a neuronal fate. Finally, cells exit the cell cycle, form dendritic connections within the existing network and undergo terminal differentiation into mature neurons.

Three distinct cell types are found in the SGZ of the dentate gyrus (reviewed by Kempermann *et al.,* 2004; **Figure 1.3**):

- Type-1 cells – these are early precursors of developing neurons. They represent an equivalent of the type-B cells found in the SVZ of the lateral ventricles and exhibit morphology similar to radial glial cells, which are triangular with long apical processes. (Filippov *et al.,* 2003). Type-1 cells express proteins such as nestin and GFAP and have electrophysiological properties similar to astrocytes (Seri *et al.,* 2001). They represent approximately 5% of the total cell divisions within the SGZ and typically divide asymmetrically to produce one type-2 daughter cell and one additional type-1 cell (Kronenberg *et al.,* 2003). Precursor cells in the DG are multipotent and produce both neurons and glial cells, although it is unclear whether both cell types originate from the same type-1 cells.
- Type-2 cells – type-1 cells give rise to these proliferating intermediate precursors, which are morphologically distinct from type-1 cells as they have much shorter processes and are orientated horizontally rather than vertically within the cellular layer (Kronenberg *et al.,* 2003). At this stage, the soma becomes distinctly reduced and the cells lack long apical processes. Proteins expressed overlap those of early differentiating precursor cells and immature neurons; earlier type 2a cells express markers such as nestin, which is indicative of early precursor cells, whilst the later type 2b cells express DCX, an immature neuronal marker (Steiner *et al.,* 2006). These cells comprise stages 2 to 4 of neurogenesis in the hippocampus and are the first steps of lineage restriction towards a neuronal fate.
- Type-3 cells – these cells express markers of a neuronal cell type such as DCX and lack markers indicative of a glial cell fate. At stage 4 of development, type-3 cells undergo a transition from neuroblast to postmitotic immature neuron, usually associated with a substantial reduction in proliferative activity (Brandt *et al.,* 2003). During stage 4, the developing neurons have processes of a variety of lengths and complexities and cells may be oriented in a range of positions. In contrast to neurogenesis in the SVZ, minimal radial migration into the granule cell layer occurs.

During postmitotic stages 5 and 6, final differentiation of the newly generated cells into functioning neurons occurs. Stage 5 lasts approximately 2 weeks and coincides with increased calretinin expression (Brandt *et al.,* 2003). Development of a complex system of dendrites and extension of the axon towards the CA3 region of the hippocampus occurs before synaptic contacts are formed. Axonal contacts are then made as early as 10 days after cell division and the first dendritic processes develop around a week later (reviewed by Ehninger and Kempermann, 2008). Calretinin expression is exchanged for calbindin at 2 to 3 weeks post-birth, signifying the final transition from stage 5 to 6. Young granule cells at this stage display unique electrophysiological properties including low activation thresholds, high resting membrane potentials and the ability to undergo long-term potentiation more easily (Schmidt-Hieber *et al.,* 2004). By 7 weeks post-division, new neurons are indistinguishable from the pre-existing neurons within the DG circuitry.

1.4.3 Quantification of adult neurogenesis

Quantification of adult neurogenesis is complex, as no single marker can be used to identify the complete process. Ultimately, detection of neurogenesis relies on combining several different labelling techniques to measure the two main stages of development: cellular proliferation and commitment of cells to a neuronal fate. Cellular proliferation only occurs during the initial stages of neurogenesis and the most effective way to identify and quantify cells at this stage is via analysis of cell cycle activity (reviewed by Kuhn and Peterson, 2008). For example, the DNA replication event that occurs during S-phase provides opportunity for integration of a thymidine analogue to quantify replication. Thymidine analogues incorporate not only into the cell during this specific stage of the cycle, but remain incorporated in the DNA of any progeny of the cell (Fujita *et al.,* 1966; Gratzner, 1982), providing an additional opportunity to track the fate of labelled cells. A commonly used thymidine analogue is 5-bromo-2-deoxyuridine (BrdU), a compound incorporated during DNA replication that can be detected using immunohistochemistry even in thick tissue sections (Scharfman *et al.,* 2005).

As the process of neurogenesis involves many distinct developmental steps, the very specific time-frame during which particular proteins are expressed provides an opportunity to identify developing neurons at many stages of their development using

endogenous markers (**Table 1.2**). Expression of endogenous proteins can be visualised *in situ* using immunohistochemistry (IHC) performed on relevant brain sections following sacrifice at the end of an experiment (Scharfman *et al.*, 2005). In the quantification of neurogenesis, once the required cellular markers have been identified, it is essential to choose a suitable technique for labelling and quantifying cells expressing the protein of interest. One of the most common approaches is secondary (indirect) immunofluorescence, which utilises two antibodies. The first is an unlabelled primary antibody that recognises and binds specifically to the target protein (e.g. DCX protein); a secondary antibody carrying a fluorophore is then incubated with the tissue and recognises the primary antibody and binds to it (**Figure 1.4**). There are several advantages to indirect immunofluorescence (Buchwalow and Bocker, 2010):

- Increased sensitivity due to signal amplification as multiple secondary antibodies can bind to a single primary antibody.
- Secondary antibodies are relatively inexpensive so the benefit of increased sensitivity is not outweighed by high financial costs.
- Quality control is generally superior in secondary antibodies.
- A greater range of conjugated fluorophore colours are available compared to the range conjugated to primary antibodies. This enhances the opportunity for labelling multiple proteins in a single tissue section.

When choosing a suitable antibody a range of factors must also be taken into account (Harlow and Lane, 1999):

- The nature of the sample – the region of the protein to be labelled can vary from individual peptides to a full length protein. Antibodies are designed to target specific regions so it is important to select an antibody raised against an antigen that is identical to the fragment or region to be detected.
- Processing method – some antibodies require a sample to be processed using a specific method; recognition of an antigen may only be possible when a protein has been denatured or reduced. Some antibodies are also unsuitable for tissue that is frozen, fixed with formaldehyde or paraffin-embedded, all of which are common processing methods prior to immunohistochemistry.

Endogenous marker	Stage of development	Marker information	Methodological considerations
Ki67	Proliferation	Ki67 is a nuclear protein associated with ribosomal RNA transcription during proliferation (reviewed by Scholzen and Gerdes, 2000). It is expressed in all active phases of the cell cycle but is absent from resting cells.	Glial, as well as neuronal lineage cells are labelled. This may lead to artificially inflated cell counts as it does not distinguish between cells that will become neurons and those that will become supporting cells.
GFAP	Proliferation	Glial fibrillary acidic protein is a marker for mature astrocytes. During neurogenesis, neurons originate from cells with astrocytic properties that express GFAP (Doetsch *et al,* 1997); targeted ablation of GFAP-positive cells in the adult mouse brain abolishes new neuron growth in both the SVZ and SGZ (Garcia *et al*, 2004). GFAP modulates astrocyte motility, is important in providing structural stability to developing cells and is up-regulated in regions of trauma or injury as part of the cellular repair process (Eng *et al.,* 2000).	GFAP expression can be found in regions undergoing repair after injury or trauma. This may lead to artificially inflated cell counts as it does distinguish between cellular repair and the normal process of proliferation.
Nestin	Proliferation	Nestin is a neural-specific intermediate filament protein expressed in cells that develop into neurons (Doyle *et al*, 2001). It is expressed in dividing cells during the early stages of development in the central nervous system (CNS) and peripheral nervous system (PNS), and although utilised as a marker of proliferation and migration very little is known about its function or regulation. It may play a complex role in regulation of the assembly and disassembly of intermediate filaments that participate in remodelling of cells (Michalczyk and Ziman, 2005).	Nestin expression in glial cells can be induced by a variety of factors, such as injury and neurotoxicity (Sahin *et al,* 1999; Yoo *et al,* 2005). This could lead to false-positive staining of cells in addition to those undergoing proliferation.
PCNA	Proliferation and fate specification	PCNA is essential for cell division. It is expressed in recently divided cells in the brain, is a cell-cycle-dependent nuclear protein that serves as a cofactor for DNA polymerase δ, and has a role in ensuring the fidelity of DNA replication (Essers *et al*., 2005). PCNA is widely distributed in the developing central nervous system including in neurogenic zones (Ino and Chiba, 2000).	Expression is modified in hippocampal neurons after global ischemia (Tomasevic *et al.,,* 1998). As a result, labelling may be influenced by developmental or physiological irregularities in the region of interest.

Dlx2	Proliferation and fate specification	The DLX proteins play a role in forebrain and craniofacial development (Eisenstat *et al.,* 1999). Dlx2 affects the proliferation of SVZ precursors by promoting the lineage transition from stem cell to transient amplifying progenitor; it enhances the proliferative response of neuronal progenitors to growth factors and is therefore a useful marker of the early stages of neurogenesis (Suh *et al.,* 2009).	The broad expression of this protein during early neurogenesis means it cannot be used to identify cells at any one specific developmental stage.
PSA-NCAM	Fate specification through to synaptic integration	PSA-NCAM (polysialic acid–neural adhesion molecule) is highly expressed during brain development. PSA-NCAM positive cells can be easily observed within the DG of the hippocampus and cells expressing this molecule typically express other known neuronal markers (Seki and Arai, 1991). Expression is upregulated in the hippocampus during learning tasks and it plays a role in promoting synaptogenesis, indicating it is a regulator of neural plasticity (Cremer *et al.,* 2000).	Stress reduces overall levels of adult neurogenesis (Luo *et al.*, 2005; Mitra *et al.*, 2006), but increases polysialylation (Sandi *et al.,* 2001). As a result, PSA-NCAM staining could be increased when neurogenesis is actually reduced.
DCX	Fate specification through to synaptic integration	Doublecortin (DCX) promotes microtubule polymerisation and is present in migrating neuroblasts and immature neurons (Francis *et al.,* 1999), directing neuronal migration by regulating the organization and stability of microtubules (Gleeson *et al.,* 1999). Expression of this protein occurs temporally with PSA-NCAM but is more specific to newly generated neurons; DCX positive cells lack antigens specific to glia or undifferentiated cells but do express early neuronal antigens (Rao and Shetty, 2004).	Not all newly generated neurons in the brain express DCX; neurons generated in the neocortex lack expression (Dayer *et al.,* 2005). As a result it may only be useful in certain regions. It identifies late neuronal precursors and early postmitotic neurons so a distinction between cell types cannot be made by DCX expression alone (Brown *et al.,* 2003).
Tuj-1	Migration and synaptic integration	Tuj-1 is a neuron-specific class III beta-tubulin expressed by newly generated immature postmitotic neurons (Menezes and Luskin, 1994). It contributes to microtubule stability in neuronal cell bodies and axons, and plays a role in axonal transport. The expression of this beta-tubulin is temporally aligned with DCX and it is often used a marker of newly generated cells in the study of neurogenesis (Gould *et al*, 2001).	Tuj-1 may only be expressed by a specific subpopulation of 'basket' cells within the DG, suggesting this marker may be unsuitable for identifying new neurons within the whole of the hippocampus (Seri *et al.,* 2004).

Calretinin	Axon targeting and synaptic integration	Calretinin is a marker of specific non-pyramidal γ-aminobutyric acid GABA-ergic neurons. This protein plays a role in many cellular functions including message targeting, intracellular calcium buffering and modulation of neuronal excitability (Camp and Wijesinghe, 2009). Neurons labelled are typically interneurons found in the olfactory bulb as well as in all layers of the hippocampus (Gulyas *et al.*, 1992). Immature neurons express calretinin after which expression switches to calbindin. Calretinin is therefore expressed during a short postmitotic window when axon targeting occurs (Ming and Song, 2005).	The short time frame of expression of this protein means only immature neurons are labelled.
Calbindin	Synaptic integration	Calbindin is a calcium-binding protein synthesised in the brain. It is expressed in mature neurons and takes over once calretinin expression is turned off. These two proteins are never expressed together (Nacher *et al.,* 2002).	This protein is only expressed in mature, integrated neurons so cannot be used to identify a complete population of newly generated neurons.
NeuN	Axon targeting and synaptic integration	Neuronal-nuclear antigen (NeuN) is expressed in most neuronal cell types throughout the adult brain. It is a soluble nuclear protein in postmitotic neurons and can be used to label both existing neurons and those newly generated (Lind *et al*, 2005).	Used alone this marker cannot distinguish between existing neurons already integrated into circuits and those newly generated during recent neurogenesis. It should therefore be combined with other markers.

Table 1.2 Outline of the range of histological markers that allow quantification of neurogenesis.
This table outlines the stage at which each protein is expressed and the primary functions of these proteins. Potential methodological issues are also discussed.

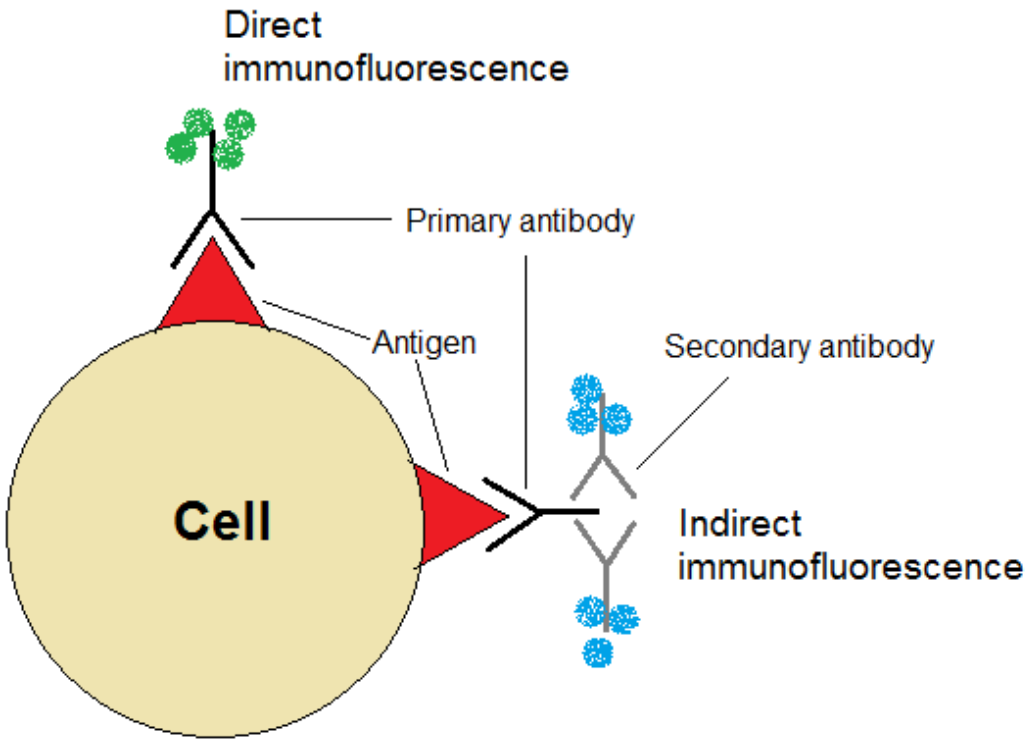


Figure 1.4 Schematic of direct and indirect immunofluorescence.
In direct immunofluorescence the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, the antibody specific for the molecule of interest (primary antibody) is unlabelled, and a second anti-immunoglobulin antibody (secondary antibody) directed towards the constant portion of the first antibody, is conjugated to the fluorescent dye.

- The species of the sample and antibody hosts – this determines reactivity between antigens and antibodies. Antibodies raised against the sample species should always be chosen. However, provided species share a large enough proportion of sequence homology in the amino acids of a particular protein, an antibody raised against a similar species could be used (e.g. rat and mouse). In this case, a prediction about the cross-reactivity of the antibody and antigen can often be made based on sequence similarity. The species of the host animal in which the primary antibody is raised is also important. The primary antibody should be raised in a species as phylogenetically distinct from the sample species as possible. This reduces the chances of cross-reactivity between the secondary antibody and the endogenous antigens in the sample. For example, when detecting antigens in mouse tissue, a primary antibody raised in a goat would be suitable. A candidate for a suitable secondary antibody would then be one raised against goat antigens in a donkey (i.e. notation for these antibodies would be: 1^0 Ab - Go α Ki67; 2^0 Ab - Do α Go).

- Whether to use polyclonal or monoclonal antibodies – polyclonal antibodies recognise multiple epitopes on any one antigen whereas monoclonal antibodies recognise only one. Detection of multiple epitopes generally leads to more robust detection and an increased signal against target proteins typically expressed at low levels. A potential drawback of using polyclonal antibody labels is that the degree of between-batch variation is greater and the lower specificity may result in increased background signal.

- Fluorophore labels – these labels are conjugated to the secondary antibody to visualise the binding of a primary antibody to a particular antigen in a sample. Positive staining indicates the presence of that protein or antigen in a cell. Fluorescent labels are commonly used as they reliably emit wavelengths of light in the visual range when excited by light of a lower, but specific, wavelength. When selecting a label, the numeric notation typically refers to the wavelength at which excitation of the fluorophore is maximal (e.g. 488 for green and 594 for red). The brand of secondary antibody with a conjugated fluorophore is also important to consider, as staining intensity and the prevalence of background staining can vary between products.

1.5 OVERALL AIMS AND OBJECTIVES

The primary aims of work presented in this thesis were to explore the factors that influence sexual signalling in male mice and how individual investment in specific signal components may influence female behaviour and neurobiology. The specific objectives were, first to investigate whether male scent marking and calling serve separate functions; both of these sexual signals are important in sexual communication but it is unclear whether they play distinct roles in communication or if individual investment in ultrasonic calls and scent marks is dependent upon factors such as social context. Additionally, the influences of female behaviour and hormones such as corticosterone and testosterone on male signalling and investment in specific scent mark components were examined (Chapter 2). The second objective was to explore the influence of male scent and investment in the male-specific pheromone, darcin, on female behaviour; more specifically, the importance of the comparative composition of individual scents for female learned preferences of locations of competing scent marks was assessed (Chapter 3). Finally, the influence of this same scent component, darcin, on adult hippocampal and olfactory neurogenesis in females was investigated (Chapter 4). To meet the overall aim, experiments combined observation of male signalling and sexual behaviour, conditioned place preference tests to assess female learning, and neurological studies using immunohistochemistry to link male signalling investment, female learning and adult neurogenesis.

CHAPTER 2: Male investment in scent marks and ultrasonic calls as sexual signals

EXPERIMENT 2

2.1 INTRODUCTION

In many species, males communicate information to potential mates using multiple sexual signals (reviewed by Bro-Jørgensen, 2010). These signals may comprise different modalities to communicate quality or identity; alternatively, multiple signals of the same modality may be used to communicate with conspecifics. Given the potential costs associated with producing and receiving sexual signals, the widespread use of multiple signals in sexual communication has been the subject of much debate. Several hypotheses have been developed to explain how a system of multiple signals might evolve and why individuals invest in multi-component signalling rather than simply concentrating on producing a single signal of quality or condition. Additionally, to identify how and why males use multiple sexual signals in attraction and courtship of mates it is important to understand the effects of a range of factors that influence the expression of these signals within a multi-component system. This may include differences in signal investment according to the context in which communication with conspecifics occurs. For example, males may increase investment in specific signals under competitive conditions or in response to subtle changes in behaviour of females during courtship; males who are attentive to these changes could reduce signalling costs by limiting time and energy expenditure or by reducing the risk of aggressive rejection.

In addition to the presence and behaviour of conspecifics, physiological mechanisms that regulate signal expression such as individual levels of specific hormones may influence male signalling investment. Testosterone has been linked to investment in a range of sexual signals. For example, the physiological structures associated with vocal signals in mammals are particularly sensitive to hormonal changes (Beckford *et al.,* 1985), and the production of some components important for olfactory signalling in rodents is thought to be influenced by androgens (Knopf *et al.,* 1983; Novotny *et al.,* 1984). Another hormone that may be important for the modulation of sexual signalling is corticosterone, a glucocorticoid steroid produced in the cortex of the adrenal gland. Corticosterone secretion is associated with the onset of stress that may occur as a result of unfavourable changes to the external environment, interactions with conspecifics or predators, or internal biological challenges such as reproduction. Stress and the associated elevation of corticosteroids such as corticosterone can have profound effects on many aspects of physiology including metabolism, cognition, and reproduction (reviewed by Touma and Palme, 2005). As a result, development or

investment in sexual signals may be affected by the response of an individual to stress. Examples include the negative feedback link between corticosterone and melanogenesis that may mediate the expression of some visual signals (Slominski *et al.,* 2004), and the corticosterone induced reduction in metabolism that could influence energetically expensive production of olfactory signal components.

To investigate why males invest in multiple sexual signals and the role of female behaviour or individual hormones in signal investment mice are a particularly useful species. Adult males invest in scent marking and ultrasonic vocalisations (USVs), both of which are sexually dimorphic and known to be influenced by social status and androgens (Desjardins *et al.*, 1973; Nyby *et al.*, 1976; Lumley *et al.*, 1999). Both signals appear to be important in sexual communication, but it is unclear whether scent marking and calling serve distinct functions or if they provide similar information regarding male quality to potential mates. In addition, the extent to which individual variation in investment in these behaviours is related to male sexual motivation and hormonal status, or to the context in which communication occurs, is unknown.

2.1.1 The evolution of multiple sexual signals

In many species, males communicate information to potential mates using multiple sexual signals. Systems involving multiple signals or traits are typically referred to as either multicomponent or multimodal. Multicomponent signals involve the same sensory modality but convey information about different aspects of quality (e.g. red colouration and horn size). Multimodal signals convey information via different sensory modalities (e.g. visual and chemical signals). Several hypotheses have been developed to explain how a system of multiple signals might evolve (Møller and Pomiankowski, 1993).

1. The multiple message hypothesis – this supposes that different sexual signals communicate different information to intended receivers. Each signal may convey information regarding different aspects of male quality, which together indicate the overall suitability of an individual as a mate. In addition, each of the sexual signals may be adapted for use in different contexts; as a result, more than one signal may be needed for communication in all contexts important for

mate choice decisions (Tinbergen, 1959). Multiple signals may also reflect different aspects of male condition, such as the size of an ornament that is influenced by food intake, and coloration of the ornament that is influenced by primary food types. An example of this is the red body and blue eye coloration of male sticklebacks; body coloration reflects infection by parasites but blue eye coloration does not (Milinski and Bakker, 1990).

2. The redundant signal or 'back-up signal' hypothesis (Johnstone, 1996) – this is based on the assumption that each sexual signal provides the same information regarding signaller quality but these signals are imperfect. As in the multiple messages hypothesis, evaluation of many traits or signals would provide a more accurate estimate of overall male quality. However, in contrast to multiple messages, the redundant signal hypothesis suggests each of the multiple signals convey information regarding the same aspect of male quality. For this hypothesis to be correct, mate choice based on assessment of a single signal would impose a risk of selecting a male that appears to be of high quality but in reality is not. As a result, the probability of selecting a mate of high quality would be improved by gaining information from multiple signals; the increased cost in time and energy of assessing more than one signal must be offset by a reduced risk of making poor mate choice decisions based on one trait. Finally, this hypothesis predicts the evolution of multiple sexual signals would be more prevalent in lekking and group living species or in those that form mating swarms. In these species, males typically aggregate in mating arenas. As a result, the time and energy spent searching for and gaining access to potential mates is reduced and assessing multiple male signals may impose minimal costs on females.

3. The unreliable signal hypothesis (Moller and Pomiankowski, 1993) – this theory postulates some male sexual signals have undergone such intense Fisherian runaway selection that they no longer accurately signal male quality. As a result, an alternative signal must be evaluated. Fisherian selection arises due to positive feedback between an arbitrary, but heritable, male sexual signal and a corresponding heritable female preference (i.e. when female choice for certain traits is not driven by the direct or indirect benefits gained as a result of a particular mating bias, but as a result of an arbitrary preference) (Fisher, 1915).

Consequently, females produce sons that possess the attractive signal and females that prefer the same attractive signal. Genetic linkage between the preference and the preferred trait results; even though females gain no direct genetic benefits from a particular mating preference, they benefit indirectly from increased mating success of offspring bearing the signal. For example, preferred male sandflies produce sons that are attractive and achieve high mating success without any apparent good-genes benefits (Jones *et al.,* 1998). The trait often becomes particularly exaggerated increasing reproductive fitness but at the cost of reduced survival rate. After many generations this selection pressure could lead to a reduction in genetic variability among males, consequently terminating female choice for male genetic qualities (Taylor and Williams, 1982).

In addition to the potential costs or benefits associated with female assessment of multiple male signals, receiver psychology may be important. Multicomponent signalling may improve signal features such as detectability, discrimination and memorability, potentially enhancing mating and reproductive success (reviewed by Rowe, 1999). Initial detection of a signal is crucial for subsequent processing and can be enhanced when components are produced together or in quick succession. The presence of a second sexual signal, even if it acts as an accessory signal, may also increase detection of the focal signal, probably by increasing the attention and awareness of the receiver (Smith and Evans, 2008). The presence of a second signal could therefore aid detectability, regardless of whether both signals provide important information regarding male quality or one signal acts to alert the receiver to the presence of the other. A good example of this is the visual tuft displayed by male wolf spiders (*Schizocosa ocreata*). Although males produce vibrations that are important for communication during attraction and courtship, the visual tufts improve detection by females as the efficacy of vibrations alone can be limited due to the complex leaf litter habitat this species occupies (Scheffer *et al.,* 1996).

Signal discrimination is particularly important when individuals need to respond appropriately to differences in the same signal produced by individual males, often because the difference between the signals indicates differences in the relative quality of the signaller. These discriminations typically occur through learned association and experiences, or through an innate pre-existing bias. When discrimination between signallers is based on one parameter of a signal (e.g. size, brightness, amplitude),

selection may favour the evolution of signals that receivers can easily differentiate. Discrimination of a signal may also be improved by the presence of a second sexual signal that has evolved to amplify the original signal. For example, the black coloration around the area of carotenoid pigment on male guppies (*Poecilia reticulate*) may amplify the adjacent orange coloration without actually being an important indicator of male quality itself (Brooks and Caithness, 1995).

Finally, signal memorability is important as it is often linked to how readily learning about that particular signal and the qualities it indicates occurs. Whilst learning typically involves understanding the associations between a sexual signal and underlying genetic quality or male condition, memory is important in maintaining that association over longer periods of time. If the intensity of signals in a multi-component system is similar, learning may be achieved more quickly when both signals can be detected together than if individual signals are perceived alone (Rowe, 1999; Candolin, 2003). Learning can also be improved if multiple signals are presented together after individuals have learned about each signal independently, or if the learning of one signal promotes the learning of another, an effect known as potentiation (Guilford and Dawkins, 1991). Potentiation is more common when the signals vary in sensory modality, suggesting females may be able to learn and assess multiple signals more quickly and reliably if males produce multiple sexual signals of different modalities. Although multiple signals are thought to convey information about distinct aspects of mate quality or lead to signal redundancy, males of some species reliably signal the same aspects of quality via two different sensory modalities. For example, male common wall lizards (*Podarcis muralis*) produce visual and olfactory signals that both reliably signal health status and parasite load (Martin *et al.*, 2008). This may reinforce the reliability of each separate signal when both are perceived simultaneously (i.e. feigning quality via one signal may be possible but feigning quality in two signals is more implausible).

2.1.2 The multiple sexual signals of mice

Adult male mice invest in two distinct sexual signals: olfactory signals that include scent marks and auditory signals in the form of ultrasonic vocalisations (USVs). Male scent marks are attractive to females and are often encountered within the environment prior to an interaction with a male. As discussed previously in section

1.3.2, the composition of scent marks signals genetic identity in several species (e.g. Lawson *et al.,* 2000; Charpentier *et al.,* 2008) and in mice, the major urinary proteins (MUPs) extend the life of a scent mark (Hurst *et al*., 1998), provide males with an individual and genetic olfactory signature (Hurst *et al.,* 2001; Cheetham *et al.,* 2007), and stimulate female sexual attraction to male urine. This attraction is mediated by the male-specific MUP, darcin, and contact with this large, involatile protein stimulates female learning of the associated individual volatile profile of a scent (Roberts *et al.,* 2010). This allows sexual attraction to remain inherent but selective towards particular males. An additional source of genetic information present in male scent is MHC odour-type. The major histocompatibility complex (MHC) is a complex set of genes that encodes glycoproteins involved in self recognition in the immune system and influences the scents produced by individuals of many species (Singh *et al.,* 1987; Penn and Potts, 1998a; Strandh *et al.,* 2012), potentially via the presence of molecule fragments or altered the peptide metabolites in urine (Singh *et al.,* 1987; Singer *et al.,* 1997). Evidence of MHC-based mating preferences has been established in a wide variety of species including rats, fish and humans (reviewed by Brown and Eklund, 1994; Jordan and Bruford, 1998; Penn, 2002), and in mice is thought to be based upon imprinting on maternal odours during the rearing period in the nest (Penn and Potts, 1998b).

In addition to providing information regarding genetic and individual identity, scent marks may signal important information about health status. Females may be more likely to produce offspring with increased disease resistance and reduce the likelihood of contracting diseases themselves if infected males are avoided. As scent marks are often encountered prior to an interaction with a male, the presence of an honest indicator of health status within a scent mark allows infected males to be avoided. Females are typically less attracted to the scents of infected individuals (Kavaliers and Colwell, 1995); in mice the ability to distinguish between healthy and infected males is amplified when males are sexually stimulated or scent mark at a high rate (Zala *et al*., 2004). Females may therefore discriminate the urine of males carrying infections or parasites and use this information to preferentially associate and mate with uninfected individuals (Ehman and Scott, 2002).

Along with MUPs and MHC-type, two sesquiterpenes are secreted by the preputial glands of males and added to urine when scent marks are deposited (Novotny *et al.,* 1990). The two compounds most commonly produced by male mice are E,E-α-

farnesene and E-β-farnesene (Novotny *et al.,* 1990). Similarly to the volatile components of mouse urine such as 2-sec-butyl 4,5 dihydrothiazole (thiazole) and 3,4 dehydro-exo-brevicomin (brevicomin) that are partially suppressed in subordinate males, expression of farnesenes is lower in these individuals that also have smaller preputial glands than dominant territory holders (Novotny *et al.,* 1990; Harvey *et al.,* 1989). These many highly volatile components found in male urine are attractive to female mice (Jemiolo *et al.,* 1985), stimulate oestrus (Ma *et al.,* 1999) and stimulate aggression between males (Novotny *et al.,* 1985).

Whilst the involatile and volatile components of scent marks signal important information including identity and health status, the location and pattern of male marks indicates territory size, and social dominance may be advertised via countermarking (Rich and Hurst, 1998). When countermarking, males deposit urine next to the marks of intruders or subordinates. This reduces ambiguity for females when attempting to establish the identity of the territory owner and allows males to respond to the challenge for dominance that the scents of other males may represent (Gosling, 1982). By countermarking all other male scents, individuals are therefore able to reinforce their dominance and ensure their scent remains the most recently deposited. This may be particularly important for female assessment of males; females can distinguish between two scent marks based on the age of the scents and prefer the scent deposited most recently (Rich and Hurst, 1999). Scent mark frequency is also important for female assessment of males as marking rate is often indicative of social status (Desjardins *et al.,* 1973), and females typically prefer dominant individuals as mates and avoid the mating attempts of subordinates (Horne and Ylonen, 1996); marking rate predicts female preferences in mice when manipulated together with genetic dissimilarity (Roberts and Gosling, 2003).

A second sexual signal produced by adult male mice is the ultrasonic vocalisation (USV). Ultrasonic calls first occur during early post-natal development when pups produce ultrasounds to elicit maternal care and indicate distress (reviewed by Portfors, 2007). Concurrent with the transition to sexual maturity, males then begin to produce ultrasonic vocalisations at a frequency of around 70 KHz when they encounter female conspecifics or female odours (Whitney and Nyby, 1979), potentially mimicking the calls produced by pups to reduce aggression or rejection by females (Whitney *et al.,* 1973). As a result, it has been suggested that ultrasound production serves a specific

courtship function in mice (Nyby *et al.*, 1977). Male ultrasound elicitation is restricted to encounters with females or female odours (e.g. urine or soiled nesting material) and the type of odour is important. For example, males do not typically respond to female urine that has been aged or urine from pre-pubertal females, but facial and vaginal secretions do elicit ultrasounds (Nyby et al., 1979; Nyby and Zakeski, 1980; Byatt and Nyby, 1986). When responding to females, the majority of male ultrasounds are emitted during the initial stages of an encounter, when mutual sniffing and grooming are observed, and typically cease following mounting and ejaculation (Nyby, 1983). The investment made by individual males in auditory signals is related to hormonal and social status. Repeat episodes of social defeat results in a short-term reduction in ultrasound production (Lumley *et al.*, 1999), while castration reduces the levels of ultrasound production and increases the latency to call; subsequent testosterone treatment is sufficient to completely restore previous calling investment (Matochik *et al.*, 1994). As females are typically more likely to associate with a vocalising male over an individual that fails to emit any ultrasounds (Pomerantz *et al.*, 1983), initial attraction and subsequent courtship may be mediated by this auditory signal.

As the ultrasonic repertoire of males differs between individuals (Holy and Guo, 2005), it is possible that females may be able to recognise individual males based on this signal. Female responses to male vocalisations in wild mice indicate a preference for the calls of non-kin over those from familiar, related individuals (Musolf *et al.,* 2010), suggesting the components of a male's call may play a role in kin recognition. However, little is known about the function of male calls in mate choice or whether calling investment provides a reliable signal of any aspects of male quality. Despite this, the androgen dependence of individual calling investment suggests this signal may be linked to other aspects of male quality that are also influenced by testosterone (Nunez *et al.,* 1978).

2.1.3 The influence of female conspecifics on male signalling

Male investment in sexual signals is important for both attraction and courtship. Courtship is typically defined as the interaction between males and females that leads to a decision about mating or mate choice, and standard models of sexual selection assume that male signals reflect quality or condition and individual investment should always be

maximal (Andersson, 1994). However, despite behaviour and signalling during sexual interactions often occurring at a relatively fixed rate or intensity, there is potential for female behavioural responses to influence male signalling investment. Males must invest in signals to attract females and maintain close proximity, but these signals may be costly to produce or be highly conspicuous, leading to increased predation risk and male competition. Conversely, females must be able to assess male quality without increasing their own predation risk as a result of spending prolonged periods of time close to males that signal intensely. As a result, it may be beneficial for males to modulate their signalling investment based on female behaviour. Continuing to signal only when females are responsive and show interest in a male, or reducing signalling once a mating opportunity has been secured, may reduce the costs of signalling unnecessarily for prolonged periods of time. Males who are attentive to female cues or behaviours could therefore reduce their signalling costs by limiting their time and energy expenditure, reducing the risk of aggressive rejections or interactions with competitors and limiting their predation risk. Alternatively, when there is little or no cost associated with producing a sexual signal at a high level of intensity, males may be expected to invest heavily regardless of female behaviour.

Male adjustments in signalling investment in response to female behaviour are common in birds, where visual and auditory displays are energetically expensive and often attract the attention of predators. For example, male whitethroats (*Sylvia communis*) court females by simultaneously diving and producing a complex song. Females produce sharp calls in response and sometimes jump towards the male. These calls and jumps influence male courtship behaviour; sharp female calls attract male attention, causing courtship performances to occur sooner, whilst female jumping increases the rate at which male songs are produced (Balsby and Dabelsteen, 2002). Male satin bowerbirds (*Ptilonorhynchus violaceus*) adjust their display rate in response to signals from females, reducing the intensity of signalling when females appear to be startled or unresponsive (Patricelli *et al.,* 2002). Evidence of male adjustments to signalling effort in response to female behaviour is less common in mammals, although female vocalisations in species such as Barbary macaques (*Macaca sylvanus*)are thought to signal receptivity, and males adjust their approach behaviour as a result (Semple and McComb, 2000). However, little is known about the influence of female behaviour on male signalling in rodents, particularly during courtship when males produce ultrasonic calls.

When measured over the first five minutes of an encounter, calling investment is thought to be relatively stable regardless of female behaviour (Whitney *et al.,* 1973). However, the consistency of male signalling over more prolonged sexual encounters in response to female behaviour is not known.

2.1.4 Hormones and male sexual signals

Hormones can have a potent influence on male courtship and signalling, acting as a physiological intermediary that modulates investment in specific sexual signals. The immunocompetence handicap hypothesis postulates that androgens such as testosterone simultaneously regulate the expression of secondary sexual signals and impose impairments on the immune system (Folstad and Karter, 1992). Males must therefore balance signalling investment and a potential reduction in immunity; only high quality males with particularly strong immune systems or genetic resistance would be able to withstand the costs of high testosterone required to produce the most attractive or intense signals. Testosterone has been linked to investment in a range of sexual signals and in birds may be linked to the expression of colourful plumage or badges, as these are often produced seasonally and coincide with changes in levels of this hormone (reviewed by Kimball, 2006). For example, in male house sparrows (*Passer domesticus*), a bib of black feathers is acquired annually in the summer after moulting. Experimental reduction of circulating testosterone reduces the exposure of the black bib in males and the white tips of the black bib feathers wear off much later in the season (Gonzalez *et al.,* 2001). Similarly, the size of male supra-orbital combs in black grouse (*Tetrao tetrix*), acquisition of bright male plumage in fairy wrens (*Malurus cyaneus*) and the intensity of the UV/blue crown colouring in male blue tits (*Cyanistes caeruleus*) are all correlated with individual testosterone levels (Peters *et al.,* 2000; Rintamaki *et al.,* 2000; Roberts *et al.,* 2009).

Testosterone is linked with other secondary sexual signals such as calling behaviour and the production of chemical cues. Vocal signals in mammals are particularly sensitive to hormonal changes. Calls are generated by converting the airflow from the lungs into acoustic energy in the larynx, which is followed by filtering in the vocal tract. Male vocal folds within the vocal tract and individual call rate are both influenced by testosterone, so auditory sexual signals may provide reliable information

about the hormonal status of an individual, which may in turn be linked to social status and fertility (Beckford *et al.,* 1985). For example, male giant pandas (*Ailuropoda melanoleuca*) produce bleats during encounters with oestrus females, which accurately reflect testosterone levels in addition to individual identity (Charlton *et al.,* 2009; Charlton *et al.,* 2011). In rodents, the effects of testosterone on the production of sexual signals are well understood. Castration leads to a significant reduction in scent mark rate and the number of vocalisations that males produce (Yahr *et al.,* 1979; Johnston, 1981; Kimura and Hagiwara, 1985); subsequent administration of testosterone is sufficient to fully restore these behaviours (reviewed by Floody, 1981). This hormone is also essential for spermatogenesis in males (Ludwig, 1950); low testosterone can result in reduced fertility and abnormal development of accessory reproductive glands important in the production of seminal fluid (Dyson and Orgebin-Crist, 1973; Shima *et al.,* 1990). As a result, androgen-dependent investment in USVs and scent marks may reflect aspects of male reproductive physiology that would be important to females when assessing potential mates such as ejaculate quality, fertility and sperm count. Further, the expression of some individual MUPs within the scent marks of mice is influenced by testosterone levels (Clissold *et al.,* 1984), such that some are male specific (Knopf *et al.,* 1983). These proteins are essential for the attraction of females to scent locations and provide not only an individual scent signature but a mechanism for allowing females to remember locations where scents are encountered (Hurst *et al.,* 2001; Roberts *et al.,* 2012).

In addition to testosterone, another hormone important for the modulation of sexual signalling is corticosterone, a glucocorticoid produced in the cortex of the adrenal gland. The rapid response of the neuroendocrine system to changes in both the internal and external environment allows animals to maintain internal homeostasis through activation of the hypothalamo-pituitary-adrenal (HPA) axis. In many species, corticosterone is the major adrenal steroid secreted as a result of HPA axis stimulation. Activation of the HPA axis is commonly associated with the onset of stress that may occur as a result of unfavourable changes to the external environment, interactions with conspecifics or predators, or internal biological challenges such as reproduction (reviewed by Touma and Palme, 2005).

The long term consequences of the stress response for reproductive fitness and survival are often unclear, but individual variation in the ability to cope with stress is

likely to impact life history traits and may be linked to investment in sexual signals. For example, some specific stress hormones such as corticosterone have a suppressive influence on the immune system (reviewed by Apanius, 1998). If stressful events coincide with the development of a secondary sexual signal, the simultaneous effects of corticosterone on signal development and immune system suppression provide a route through which the immunocompetence handicap theory of sexual signal evolution could operate; only high quality males with particularly strong immune systems or genetic resistance would be able to withstand the costs of high corticosterone during signal development. Alternatively, condition-dependent sexual signals may be directly affected by the stress response of an individual without suppression of the immune system. Short-term release of stress hormones is adaptive, reallocating energy to tasks that are essential for survival and restoration of homeostasis (Charmandari *et al.,* 2005). However, prolonged high levels of these hormones may be detrimental to survival and can have profound effects on many regions of the body, including the hippocampus and the CNS (Murray *et al.,* 2008), which may ultimately impact learning and the development of signals that have a neural basis such as bird song. In support of this, increased levels of corticosterone are correlated with reduced call complexity in some bird species. For example, male zebra finches (*Taeniopygia guttata*) with elevated levels of plasma corticosterone develop songs with significantly lower complexity and shorter duration (Spencer *et al.,* 2003). Elevated levels of corticosterone may therefore have a negative influence on the development of some secondary sexual traits.

There is evidence that the expression of some visual or chemical signals may also be mediated by corticosterone. In the case of visual sexual signals, this may be due to a negative feedback link between corticosterone and melanogenesis (Slominski *et al.,* 2004). For example, barn owls (*Tyto alba*) show a high degree of inter-individual variation in the melanin based pigments on their feathers and males with high levels of melanin pigments typically have greater reproductive success (Roulin *et al.,* 1998; Roulin *et al.,* 2001). Male barn owls with corticosterone-releasing implants show a reduction in the degree of melanism in their feathers, suggesting expression of this visual signal is influenced by individual glucocorticoid levels (Roulin *et al.,* 2008). This effect is also observed in males of other bird species that express colour-based visual signals to attract mates (Griffith *et al.,* 1999; Fargallo *et al.,* 2007). In the case of chemical or olfactory signals, stressful events including aggressive encounters may result in

corticosterone release and a subsequent reduction in the production of chemical signals such as scent marks. A relationship between stress, corticosterone secretion and reduced scent marking has been observed in several species suggesting that prolonged elevation of adrenal hormones may impact the rate at which animals are able to synthesise and deposit important chemical signals (Lumley *et al.,* 1999; Watson *et al.,* 1999; Yamaguchi *et al.,* 2005). In rodents, the production of many scent signal components and pheromones is energetically expensive and often requires multiple processes to synthesise specific components. As a result of increased metabolic demands during periods of prolonged corticosterone elevation (Laugero and Moberg, 2000), the stress response may impact production of olfactory signals. This may in turn influence the responses of females when assessing the scents of individual males; in support of this, the normal preference female mice exhibit for male scent is significantly reduced when males are administered with corticosterone (Kavaliers and Ossenkopp, 2001).

2.1.5 Aims and Objectives

The overall aim of this study was to investigate why male mice invest in multiple sexual signals and how social context, female behaviour or individual hormone levels influence signal investment. In the first experiment, investment in scent marking and calling was recorded in a range of contexts to potentially address why males produce two different sexual signals and how investment according to social context may be influenced by individual testosterone levels. In addition, the potential links between female behaviour and male signalling during sexual interactions were investigated. The specific objectives were to answer the following questions:

1. **Is male signalling investment influenced by the context in which female cues are encountered?**

   Both male signals appear to be important in sexual communication, but it is unclear whether scent marking and calling investment vary according to the social context in which communication occurs. Here, this was addressed by recording male signalling investment in response to female odours, a female behind a mesh barrier, a direct interaction with a female or no female cues.

2. **Are individual testosterone levels linked with male signalling in different social contexts?**

   Quantitative differences between individuals in testosterone levels may be linked to differences in signal investment in a range of social contexts. To determine whether male scent marking or calling are linked to individual testosterone levels, the urinary testosterone concentration of subjects was measured prior to the experiment and the relationship between levels of this hormone and male signalling in each social context assessed.

3. **Is the expression of male urinary protein linked to scent mark investment in different social contexts?**

   Urinary protein concentration of males is indicative of MUP expression, as over 99% of protein present in mouse urine comprises MUPs (Humphries *et al.,* 1999). As scent marks and scent mark components may be energetically costly to produce (Gosling *et al.,* 2000), males may need to balance protein investment with scent mark rate. The urinary protein concentration of males was measured to assess how protein expression may be linked to scent mark investment in different social contexts.

4. **Is male signalling investment during sexual interactions linked with the behaviour of males and/or females during interactions?**

   To investigate the potential links between male signalling investment and male and female behaviour during interactions, in addition to measuring scent marks and call rate, the total duration of expression of affiliative behaviours (e.g. sniffing and following the other animal) and mounting activity of males was recorded. The links between signal investment and behaviour were then analysed.

The second experiment was conducted to extend the findings of interaction trials in the first experiment; as a result of single sexual interactions in experiment one, only correlations between male signalling investment and the behaviour of females could be identified. In experiment two, subjects participated in multiple sexual interactions to establish whether male investment in signalling is a stable trait or likely to be influenced by the behaviour of different females. As in experiment one, in addition to measuring the number of scent marks and call rate, during interactions the total duration of expression of affiliative behaviours (e.g. sniffing and following the other

animal) and male mounting activity was recorded. Additionally, the links between corticosterone, an important stress hormone, and male investment in sexual signals was examined. The specific objectives of experiment two were to answer the following questions:

1. **Is male signalling investment or behaviour of males and females consistent across multiple interactions?**

   To address this question, male signalling and behaviour was measured during three interactions with different females. For each interaction, males were ranked according to scent mark and calling investment to assess whether individual signalling was consistent across multiple interactions relative to other males; further, to explore how experience may modulate sexual motivation and signalling, affiliative behaviour and signal investment of the group was compared across the three interactions.

2. **Is male calling investment a reliable indicator of sperm count or reproductive physiology?**

   Although male calling is a specific response to female cues and is likely to play a role in courtship, whether it has any function in mate choice is unknown. As testosterone is important for fertility and normal reproductive development in males (McLachlan *et al.,* 1996), investment in a testosterone-mediated behaviour such as calling may be linked to male sperm count and specific measures of reproductive physiology. Male sperm count was recorded at the end of the experiment to assess whether calling investment during interactions is correlated with this potential measure of male quality.

3. **Is male investment in major urinary proteins linked to preputial gland or seminal vesicle size?**

   Pheromonal secretions from the male preputial gland are added to urine during scent marking (Bronson and Caroom, 1971), and the seminal vesicles are accessory glands important in the production of seminal fluid proteins (Bradshaw and Wolfe, 1977). In the context of sexual interactions with females, investment in proteins important for pre-copulatory competition, such as MUPs, may be balanced against investment in proteins associated with post-copulatory sperm competition, such as those produced in the seminal vesicles. At the end of the experiment, the weight of the preputial glands and seminal vesicles of subjects was recorded. Potential

correlations between the size of these male accessory glands and urinary protein expression were analysed to assess whether investment in urinary and accessory gland proteins is linked.

4. **Is male signalling or behaviour linked to individual levels of the stress hormone corticosterone?**

   Corticosterone is a major steroid hormone produced during the stress response, thus, individual males that have higher baseline levels of corticosterone may exhibit reduced sexual motivation and invest less in sexual signals. To assess whether corticosterone is linked to male behaviour and signalling during courtship, faecal corticosterone levels of male subjects were measured before and after each sexual interaction. Any correlations between faecal corticosterone and male scent marking, calling or sexual behaviour were then identified.

EXPERIMENT 1

In the first experiment, investment in scent marking and calling was recorded in a range of contexts to determine whether males invest in two signals to communicate to females in different social contexts. In addition, the links between male signalling and individual testosterone levels or the behaviour of females during sexual interactions were investigated.

2.2 METHODS

2.2.1 Subject and stimulus animals

Subjects were 18 male CD-1 laboratory mice obtained from an approved supplier (Harlan, UK) at 3-4 weeks of age. At 3-4 weeks mice were housed in same-sex pairs in 48cm x 11.5 cm x 12cm cages (M3, North Kent Plastics Ltd., UK). Pairs had to be separated upon animals reaching sexual maturity at approximately 12 weeks of age due to aggression between males. Individuals were then housed singly in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK). All males were 12 months old at the start of the experiment. Stimulus animals were 36 adult female C57BL/6JOlaHsd laboratory mice aged 12 months that were either obtained from an approved supplier (Harlan UK) at 3-4 weeks of age or bred in house (parents obtained from Harlan UK). At 3-4 weeks mice were housed in same-sex pairs in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK) or in same-sex groups of four in 45cm x 28cm x 13cm cages (MB1, North Kent Plastics Ltd., UK); pairs and groups were maintained throughout the study.

CD-1 males and C57BL/6 females were chosen for two main reasons. Firstly, of the strains available in the colony (CD1, BALB/c and C57BL/6), CD-1 males produced ultrasonic calls the most reliably when presented with females during pilot tests with individuals different to those used in this experiment; in the majority of cases, C57BL/6 and BALB/c males failed to vocalise even in response to female conspecifics. Secondly, individuals within each strain are genetically identical, thus, females of a different strain to subjects were chosen to minimise male familiarity with female cues. All animals used in this experiment were naïve in terms of sexual experience but had previous experience of conspecific odours. Soiled male nesting material was also added

to the home cages of females three days prior to each trial to induce oestrus (Cheetham *et al.,* 2007). Throughout the experiment all animals were maintained on a reversed 12:12 light cycle (lights off at 08:00h) and on Corn Cob Absorb 10/14 substrate with paper wool bedding material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, MO, USA). Acrylic tunnels (5cm x 15cm) and red plastic houses (Techniplast, NJ, USA) were provided for home cage enrichment.

2.2.2 Experimental procedure

All tests took place during the dark phase of the light cycle, under red lighting at a consistent level of illumination and location, and in clean test arenas measuring 45cm x 28cm x 13cm (MB1, North Kent Plastics Ltd., UK). Animals were all handled and transferred between test arenas and home cages using clear acyclic tunnels to minimise stress and anxiety during testing (Hurst and West, 2010). A clear plastic barrier with a small mesh section was present during all trials excluding interactions, thus males were confined to one side of the arena in an area measuring 45cm x 14cm x 13cm. Subjects were familiarised to a clean test arena 24h prior to the first trial. All trials lasted 30 minutes and took place in a room separate from the rest of the colony. Benchkote paper (Fisher Scientific, Nottingham, UK) was placed on the base of the test arena to assist in the quantification of scent marks. Male subjects participated in four trials in a pseudo-balanced order with 48 hours between each trial (**Figure 2.1**). Each female was used as a stimulus animal no more than twice over the course of the experiment and males were never presented with cues from a particular female more than once.

Trials were as follows:

- Control trial – The male subject was placed alone in one side of a clean test arena.

- Odour Trial – Only female odours were present. A female stimulus animal was placed into one side of the test arena for 30 minutes and then removed. The male subject was then placed into the same side of the same arena.

- Barrier Trial – A clear plastic barrier with a small mesh section was used to separate the male subject and female stimulus animal. The barrier maintained

olfactory, visual and acoustic contact between the animals but prevented sexual contact.

- Interaction Trial – Male subjects interacted freely with a female.

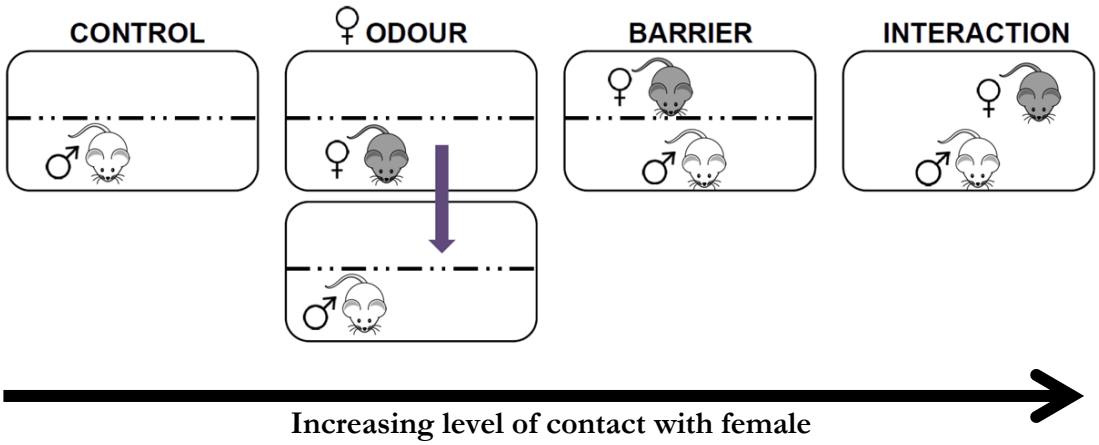


Figure 2.1 Experimental design for trials with males in four contexts: control, female odour, barrier and interaction.

2.2.3 Scent mark analysis

Scent marks deposited by males were identified by scanning the Benchkote substrate from each trial under ultraviolet light. Marking patterns were obtained using a Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and PDQuest software (Version 7.1.1, Bio-Rad) with parameters adjusted for mouse urine (10s exposure duration, 530DF60 filter, UV light source Epi illumination, high-resolution mode). Scent marks were then quantified using the 'Analyze Particles' tool in ImageJ version 1.42. Male scent marks were distinguished from those deposited by females in 'odour' and 'interaction' trials using Ponceau protein stain (Sigma Aldrich, UK), which was made up to a 0.1% solution (0.1g Ponceau solid in 100ml of 5% acetic acid) and diluted with distilled water to a 1 in 2 dilution. As the urine of male mice contains a higher total concentration of protein than urine of female mice (Cheetham *et al.*, 2009), when stained, male scent marks appeared dark pink in colour whilst female urine marks appeared pale pink/off-white (**Figure 2.2**).

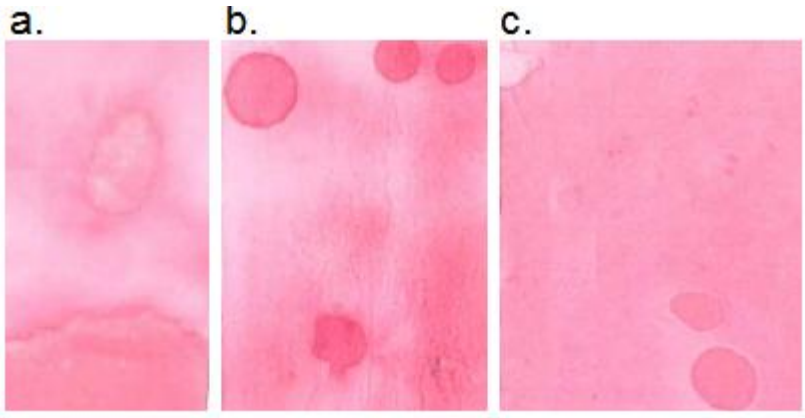


Figure 2.2 Male and female urine marks following staining with 0.1% Ponceau protein stain.
Representative examples of staining of (a) a female urine mark, which appears pale pink, (b) male urine marks, which are stained dark pink, and (c) the difference in staining between the two marks on a single sheet of Benchkote paper.

2.2.4 Urine collection

Urine was collected from male subjects 24h before the start of the experiment by holding individuals by the scruff of the neck over a clean 1.5 ml Eppendorf tube. In cases where males did not urinate immediately, gentle bladder massage was used, and this sometimes resulted in the production of urine. Samples were stored at -20°C until they were analysed for testosterone, protein and creatinine concentration.

2.2.5 Measurement of urinary protein and creatinine concentration

As testosterone and protein concentration varies with the volume of urine eliminated, the ratio of protein or testosterone to creatinine is routinely measured to correct for urine dilution (Beynon and Hurst, 2004). Creatinine is a by-product of muscle metabolism and is eliminated almost exclusively in urine. The conversion of creatine to creatinine is a non-enzymic process that proceeds at a constant rate; animals with high muscle mass excrete more creatinine in their urine such that urinary creatinine output is proportional to body mass. The protein or testosterone to creatinine ratio thus provides a simple correction for urine dilution. Measurements of raw urinary protein concentration and protein concentration adjusted for urinary dilution were both used in subsequent analyses.

Protein and creatinine concentrations were measured using assay methods previously validated for mouse urine as described by Cheetham *et al.* (2009). Protein concentrations were determined using the Coomassie plus protein assay reagent kit from Perbio Science UK Ltd. (Northumberland, UK). A standard curve was generated from a stock solution of bovine serum albumin (BSA) (1mg/ml diluted to the range 0–50µg/ml with distilled water). Each sample was diluted (typically 1:500), 100µl aliquots pipetted in duplicate to a 96 well microtitre plate and 250 µl Coomassie reagent added. After a 5 minute incubation period at room temperature, the absorbance of each sample was read at 595nm in a Labsystems iEMS-MF plate reader. A standard curve was produced using the Genesis software and the concentration of each unknown sample calculated by interpolation.

Urinary creatinine values were measured by the alkaline picrate assay from Sigma Chemicals, UK. A standard curve was generated from a stock solution of

creatinine (3mg/dl diluted to the range 0–30µg/ml with distilled water). Each sample (typically 100µl of 1:100 dilution of urine) was prepared and added in duplicate to a 96 well microtitre plate, together with 150µl of alkaline picrate reagent (5ml picrate solution: 1ml sodium hydroxide). Following a 30 minute incubation period at room temperature, absorbance at 492nm was read in a Labsystems iEMS-MF plate reader. A standard curve was produced using the Genesis software and the concentration of each unknown sample calculated by interpolation.

2.2.6 Measurement of urinary testosterone

Testosterone concentration was measured using enzyme immunoassay methods previously validated for mouse urine (Munro *et al.* 1991; Muir *et al.* 2001). Testosterone was obtained from Sigma chemicals, UK, and antibodies to testosterone and corresponding horseradish peroxidise conjugates were obtained from the Department of Population Health and Reproduction at the University of California, USA. NUNC Maxisorb plates were coated in 50µl of antibody stock diluted 1:10,000 in a coating buffer (50mmol/l bicarbonate buffer, pH 9.6) then stored for 12-14 hours at 4°C. Wash solution (0.15mol/l NaCl solution containing Tween 20) then rinsed away any unbound antibody. 25µl of phosphate buffer (0.1mol/l sodium phosphate buffer, pH 7.0 containing 8.7g NaCl and 1g BSA), 50µl of standard or sample (urine samples were diluted 1:10 in phosphate buffer), then 50µl of testosterone horseradish peroxidase (diluted 1:25,000) were added to wells. Plates were incubated at room temperature for 2 hours before rewashing. 100µl of substrate solution (Citrate buffer, H_2O_2 and 2,2'-azino-bis) was added and left to incubate at room temperature until the optical density of blank wells (i.e. those containing 0pg testosterone standard) reached a reading of one when read at 405nm, approximately 45 minutes. Plates were read with a single filter at 405 nm. Urinary creatinine was used to correct for dilution of each sample.

2.2.7 Measurement of ultrasonic calls

Ultrasonic calls were detected during all trials using a Pettersson D240x Ultrasound detector (Pettersson Elektronik AB, Sweden) set to 70 KHz, the frequency at which male mice are known to emit calls in the presence of females (Whitney and

Nyby, 1979). The detector was suspended above the cage in which trials took place out of view of animals, and headphones were attached to the detector so calls could be detected, thus minimising disturbances that may affect the behaviour of subject and stimulus animals. The heterodyne function on the detector was selected so the ultrasonic calls could be converted to a frequency within the audible range for human hearing. The amount of time males spent producing calls was recorded as an indicator of investment. This variable was chosen as a similar but more suitable method to the variable most commonly used for quantifying ultrasonic calls. Traditionally, experimental trials are divided into 5s blocks and the number of blocks containing calls is recorded (Nyby *et al,* 1977). However, this method fails to distinguish between males producing calls regularly but intermittently, and males producing calls constantly over the course of a trial. Time spent calling was therefore chosen as a more suitable indicator of individual investment. All trials were recorded remotely on DVD; detection of calls was signalled to the camera by moving a pointer over a sign labelled 'USVs' for the whole duration of the time when calls were being emitted. This was out of view of the subject animals to minimise disruption to behaviour. DVD recordings were then transcribed using an event recording program (written by Prof. R Beynon, Protein Function Group, University of Liverpool) to record when calls were heard as indicated to the camera. The transcribed data were subsequently decoded using an SPSS syntax (written by Prof. J Hurst, Mammalian Behaviour and Evolution Group, University of Liverpool). This syntax translates information recorded by the event recording program by adding together the duration of each time the pointer was moved to indicate calls were heard, thus providing a total for the time males spent calling during each 30 minute interaction.

2.2.8 Analysis of behaviour during interactions

The behaviour of subjects in each of the interactions was recorded remotely on DVD. DVD recordings were then transcribed blind relative to the signalling investment of each male. The same event recording program used to record male calling was also used to record a range of male and female behaviours. Female behaviours recorded were: total duration of sniffing male facial area, sniffing male genitals, grooming the male and following the male and the number of times females fled from a mounting

attempt. Male behaviours recorded were: total duration of sniffing female facial area, sniffing genitals of the female, grooming the female and following the female, as well as latency to first mounting attempt and number of mounting attempts. Transcribed data were subsequently decoded using a similar SPSS syntax to that used to decode male calling behaviour.

2.2.9 Data analysis

All statistical tests were carried out using the SPSS software package, version 20. Scent marking and calling data could not be normalised (assessed by Kolmogorov Smirnov and Shapiro Wilks tests $p < 0.05$), so differences in signalling investment between trials were examined using Friedman tests. Behavioural data from interaction trials were analysed using a separate principal components analysis for females and males, allowing the main patterns of behaviour to be identified. Only components with an Eigenvalue of one or greater were used in subsequent analyses as an Eigenvalue greater than 1 indicates that the component itself accounts for more variance than is accounted for by any one of the original variables in the data. Due to the non-normal distribution of the data, Spearman's rank correlations tested for relationships between signalling investment and urinary testosterone, protein and the behaviour patterns derived from the principal components analysis.

2.3 RESULTS

2.3.1 Is male signalling investment influenced by the context in which female cues are encountered?

Adult male mice invest in scent-marks and ultrasonic calls as sexual signals. Both signals are important in sexual communication but it is unclear whether scent marking and calling serve distinct functions. To address this, male scent marking and calling was recorded in response to: female odours (natural deposits), a female behind a mesh barrier, a direct interaction with a female or no female cues. The number of scent marks deposited by males differed significantly depending upon the female cues encountered (Friedman test: $\chi^2(3) = 16.7$, $p < 0.001$; **Figure 2.3a**); males typically deposited the greatest number of scent marks when separated from females by a barrier or when only female odours were present. As shown in the examples (**Figure 2.4**), males typically deposited multiple, small scent marks during 'odour' and 'barrier' trials. As many of these small marks overlap in their coverage of the substrate, the values obtained using methods described in section 2.2.3 are likely to produce underestimates of scent mark number. As a result, scent mark investment in these contexts may have been greater than shown in **Figure 2.3a**. The amount of time males spent producing ultrasonic calls also differed significantly depending upon the type of female cues encountered (Friedman test: $\chi^2(3) = 27.7$, $p < 0.001$; **Figure 2.3b**). However, the cues that elicited the highest level of calling differed from those that typically elicited the greatest amount of scent marking. Males did not call during control trials, but when presented with cues that did elicit ultrasonic calls, male calling was lowest in response to female odours and greatest during sexual interactions; calling differed significantly across the trials that did elicit calls (Friedman test: $\chi^2(2) = 10.2$, $p = 0.004$).

Male investment in scent marking and calling has been linked to a number of factors. For example, dominant individuals typically vocalise and scent mark at a higher rate than subordinates (Nyby *et al.*, 1976; Desjardins *et al.*, 1973), and repeat episodes of social defeat result in a decrease in both scent-marking and ultrasound production (Lumley *et al.*, 1999). Males that typically invest heavily in scent marking may therefore also invest heavily in calling.

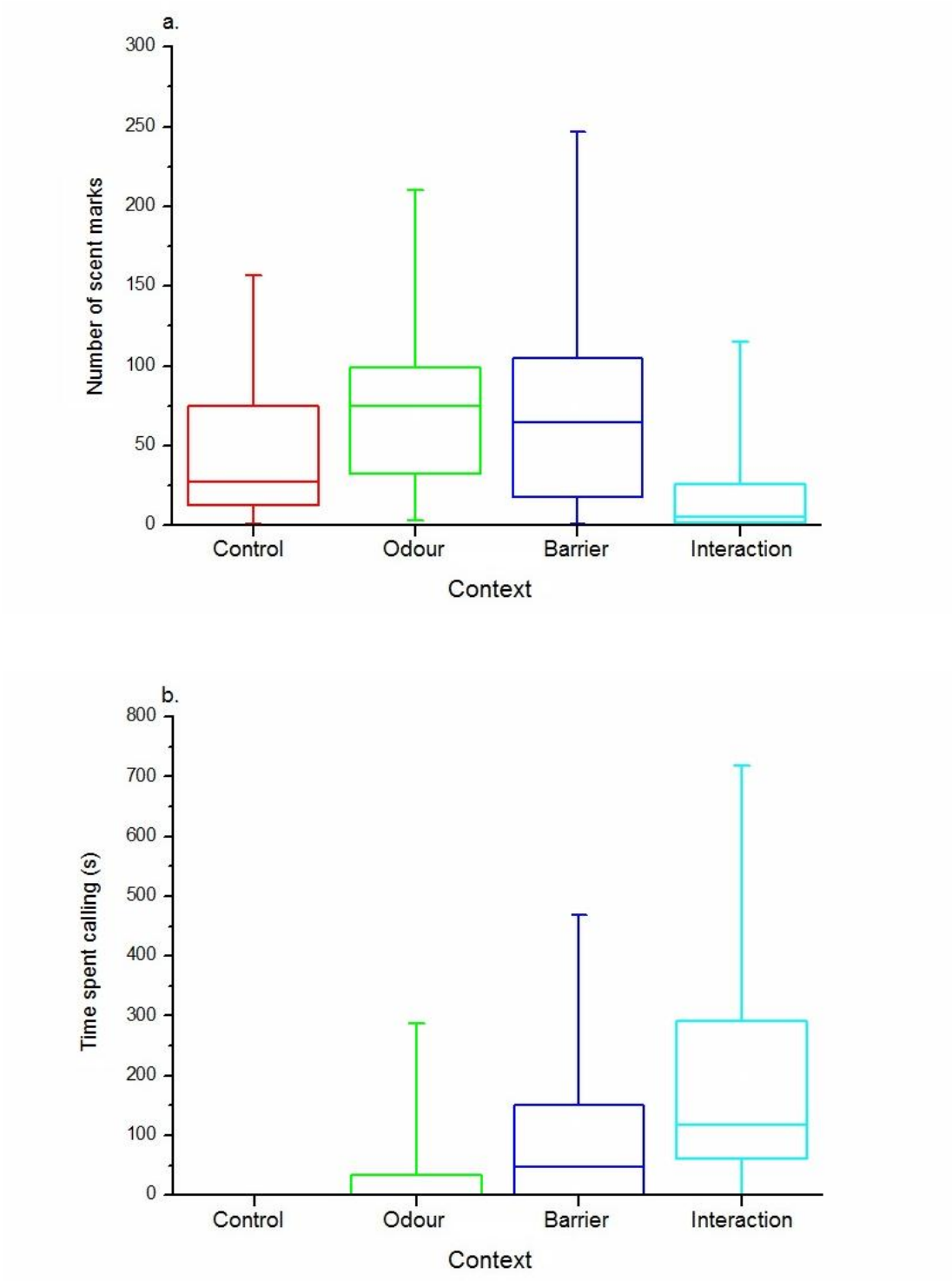


Figure 2.3 Male signalling in different communicative contexts.
Box plots (displaying medians, quartiles and 95% confidence intervals) showing male (a) scent marking and (b) calling in response to no female cues (*red*), female odour (*green*), a female behind a mesh barrier (*dark blue*) or an interaction with a female (*light blue*). n = 18 for control, odour and barrier trials, n = 17 for interactions. Male investment in scent marking and calling differed significantly according to context (scent marking: $\chi^2(3) = 16.7$, $p < 0.001$; calling: $\chi^2(3) = 27.7$, $p < 0.001$).

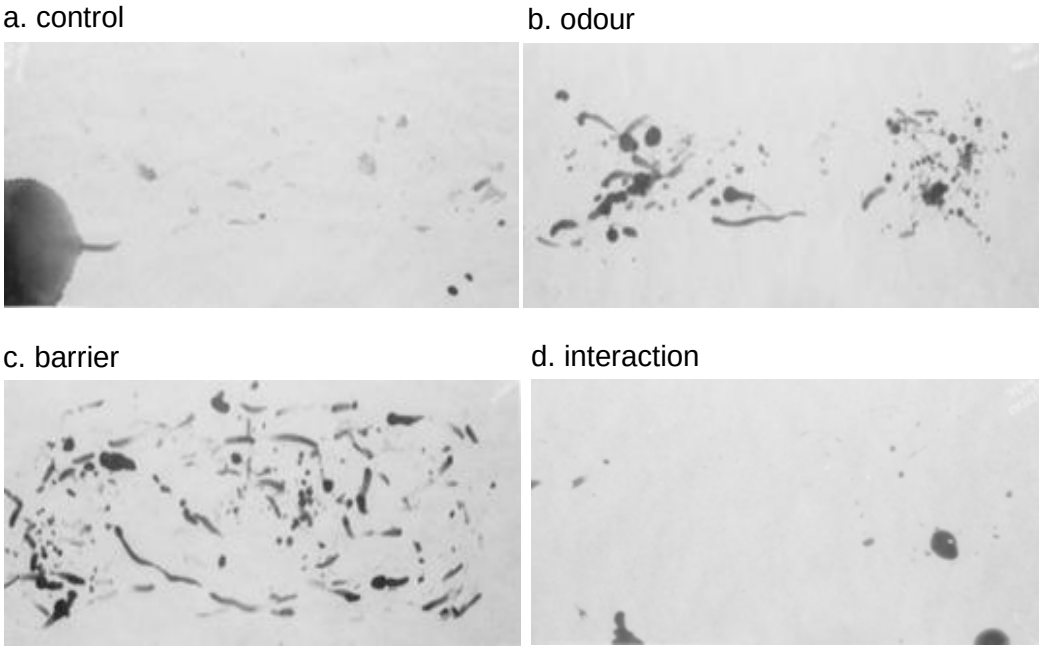


Figure 2.4 Male scent mark patterns.
Typical patterns of male scent marking in response to (a) no female cues, (b) female odour, (c) a female behind a barrier and (d) an interaction with a female.

In this experiment, male investment in both scent-marking and calling depended on the type of female cues encountered. However, cues that elicited the greatest level of scent marking differed from those that typically elicited the greatest time spent calling. Male calling was greatest during interactions with females and scent marking was highest when males were either separated from females by a mesh barrier or exposed to female odours. To determine whether individual investment in scent marking and calling was correlated, the relationship between scent marking during 'barrier' or 'odour' trials and calling during interactions was analysed. No significant correlations were found (Spearman's rho: odour: r_s = -0.09, p = 0.72; barrier: r_s = -0.12, p = 0.65). When separated from females by a barrier, male calling investment was lower than during interactions but still relatively high. In this context, when both scent mark investment and calling were relatively high, the number of scent marks was positively correlated with male calling (Spearman's rho: r_s = 0.68, p = 0.002; **Figure 2.5**). In this context, males investing heavily in calling also invested heavily in scent marking.

2.3.2 Is male testosterone correlated with male signalling in different social contexts?

Although different cues elicited the highest levels of calling and scent marking, there is evidence to suggest both signals are androgen-dependent. When castrated, males typically call and scent mark at a lower rate than intact individuals; testosterone implants are able to restore both behaviours to pre-castration levels (Matochik *et al.*, 1994). To establish how individual hormone levels may be linked to investment in sexual signalling, urine samples were collected from all subjects prior to the experiment and the testosterone concentration analysed. Male testosterone levels were positively correlated with the number of scent marks deposited (Spearman's rho; r_s = 0.57, p = 0.013; **Figure 2.6a**) and the amount of time males spent producing calls (Spearman's rho; r_s = 0.57, p = 0.014; **Figure 2.6b**), but only when males were separated from a female by a barrier (**Table 2.1**). Although multiple tests were carried out to explore the links between these factors, a Bonferoni correction was not applied due to the exploratory nature of these analyses and the risk of type II error. However, as this increases the risk of subsequent type I error, these observed correlations should be interpreted with caution.

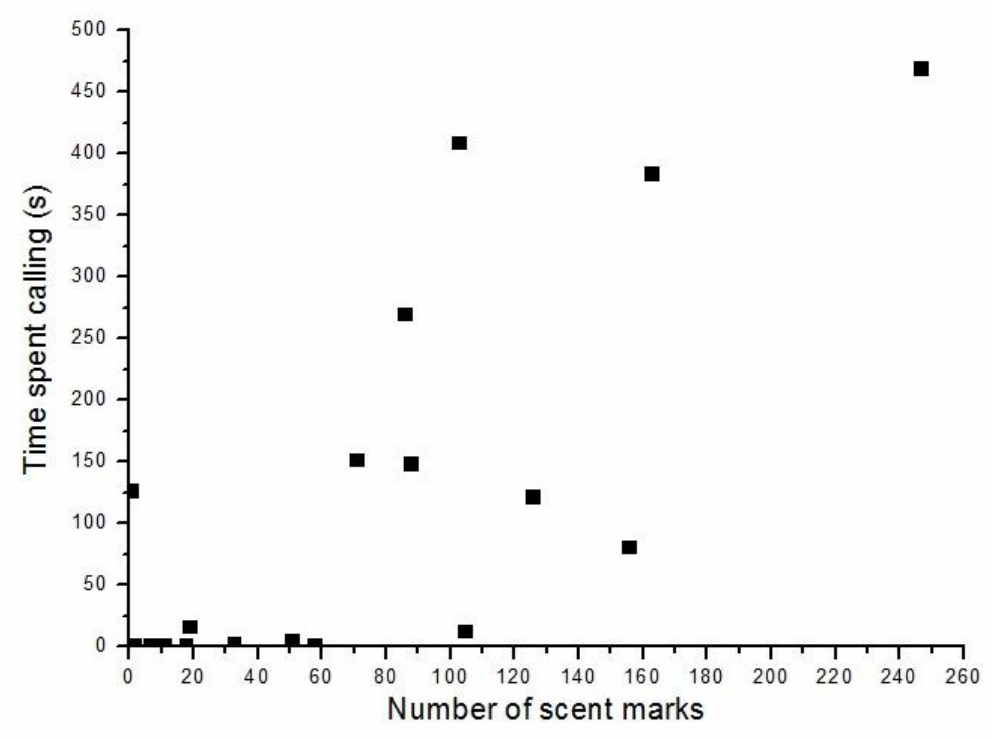


Figure 2.5 Male investment in calling and scent marking when separated from females by a barrier.

In this context, there was a significant correlation between scent marking and calling (r_s = 0.68, p = 0.002); individual males that invested heavily in scent marking also invested heavily in calling when separated from a female by a barrier.

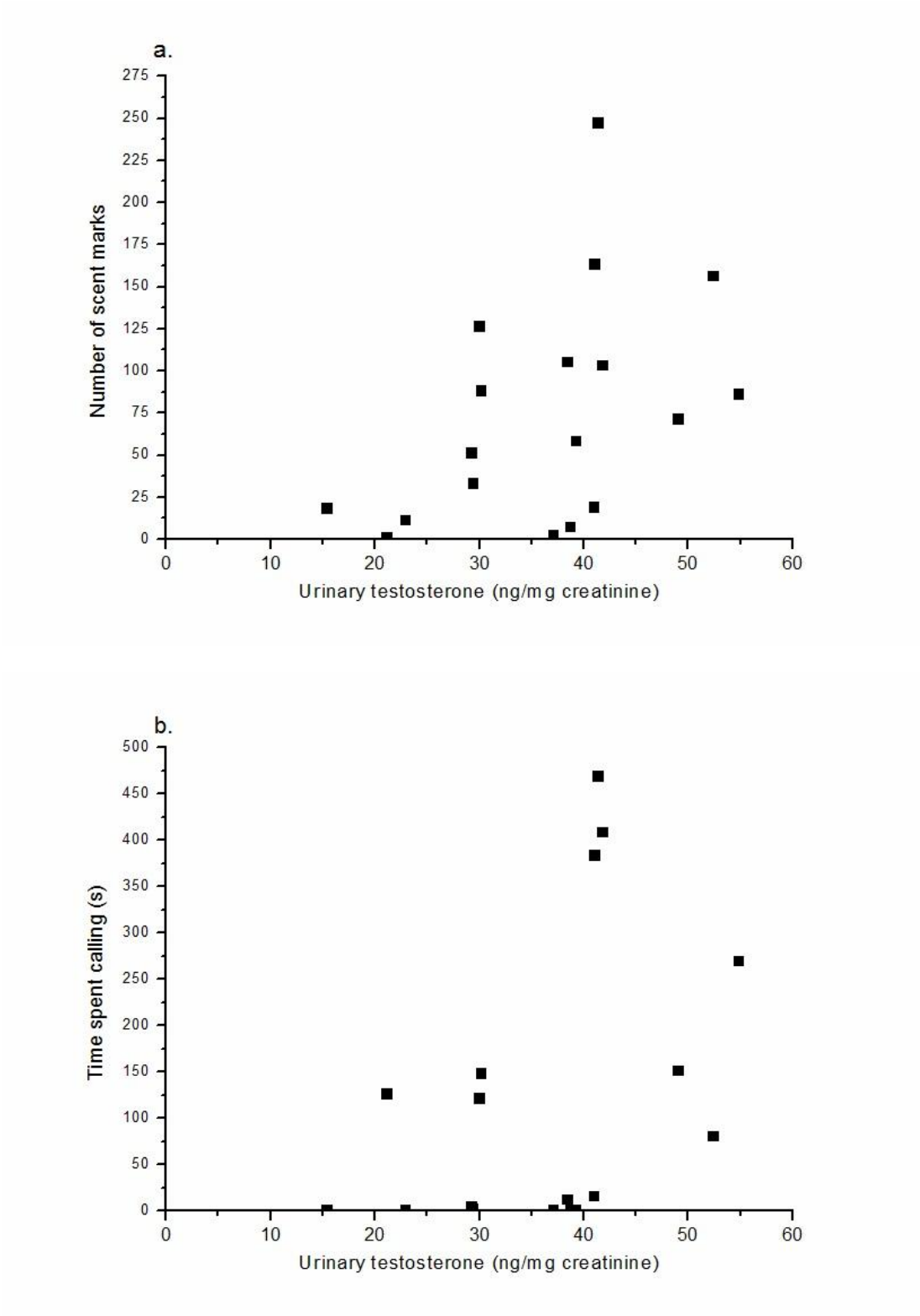


Figure 2.6 Male baseline testosterone and individual investment in signalling when separated from females by a barrier.
Testosterone was positively correlated with both (a) scent marking and (b) calling (scent marking: $r_s = 0.57$, $p = 0.013$; calling: $r_s = 0.57$, $p = 0.014$).

	Context	Spearman's rho, r_s	P value
Testosterone and scent marking	Control	0.24	0.34
	Odour	0.42	0.08
	Barrier	**0.57**	**0.013***
	Interaction	0.21	0.42
Testosterone and calling	Control	-	-
	Odour	0.06	0.82
	Barrier	**0.57**	**0.014***
	Interaction	0.27	0.30

Table 2.1 Analysis of links between testosterone (adjusted for urinary dilution) and male signalling.

Urinary testosterone was only correlated with male scent marking or calling when males were separated from females by a barrier. No data is provided for the relationship between testosterone and calling under control conditions as all males failed to emit calls during these trials. * $p < 0.05$.

2.3.3 Is the expression of male urinary protein linked to scent mark investment in different social contexts?

The urine of mice contains a high concentration of protein, of which MUPs are the major constituent. MUPs extend the life of a scent mark (Hurst *et al.*, 1998) and provide males with an individual olfactory signature (Hurst *et al.,* 2001). The pattern of MUPs expressed by individual males therefore provides important information to females when assessing the suitability of a male as a mate (Hurst, 2009). Whilst scent marking rate can be influenced by factors such as testosterone levels or social status (Desjardin *et al*, 1973; Matochik *et al.*, 1994), and under competitive conditions males typically increase scent marking and expression of MUPs (Garratt *et al.,* 2012), it is unclear whether urinary protein concentration is also linked to scent mark investment. In this experiment, urinary protein concentration was negatively correlated with the number of scent marks deposited by males (Spearman's rho; r_s = -0.48, p = 0.046; **Figure 2.7a**), but only in response to females behind a barrier. Males with low urinary protein concentration deposited a higher number of scent marks than those with high urinary protein levels. However, when protein concentration was adjusted for urinary dilution, this relationship was no longer significant (Spearman's rho; r_s = 0.18, p = 0.47); further, the number of scent marks deposited during barrier trials was negatively correlated with creatinine, a measure of urine dilution (Spearman's rho; r_s = -0.53, p = 0.03; **Figure 2.7b**). This suggests that males with more dilute urine (low creatinine concentration) deposited more scent marks.

2.3.4 Is male signalling investment linked with the behaviour of males and/or females during interactions?

The occurrence of ultrasonic vocalisations during sexual interactions is closely related to male sexual arousal in mice (Nyby, 1983) and females typically prefer to associate with males that vocalise at a high rate over individuals that fail to emit any ultrasounds (Pomerantz *et al.*, 1983). To determine whether the motivation of individuals to interact was linked with male signalling investment, the behaviour of males and females when separated by a barrier was recorded.

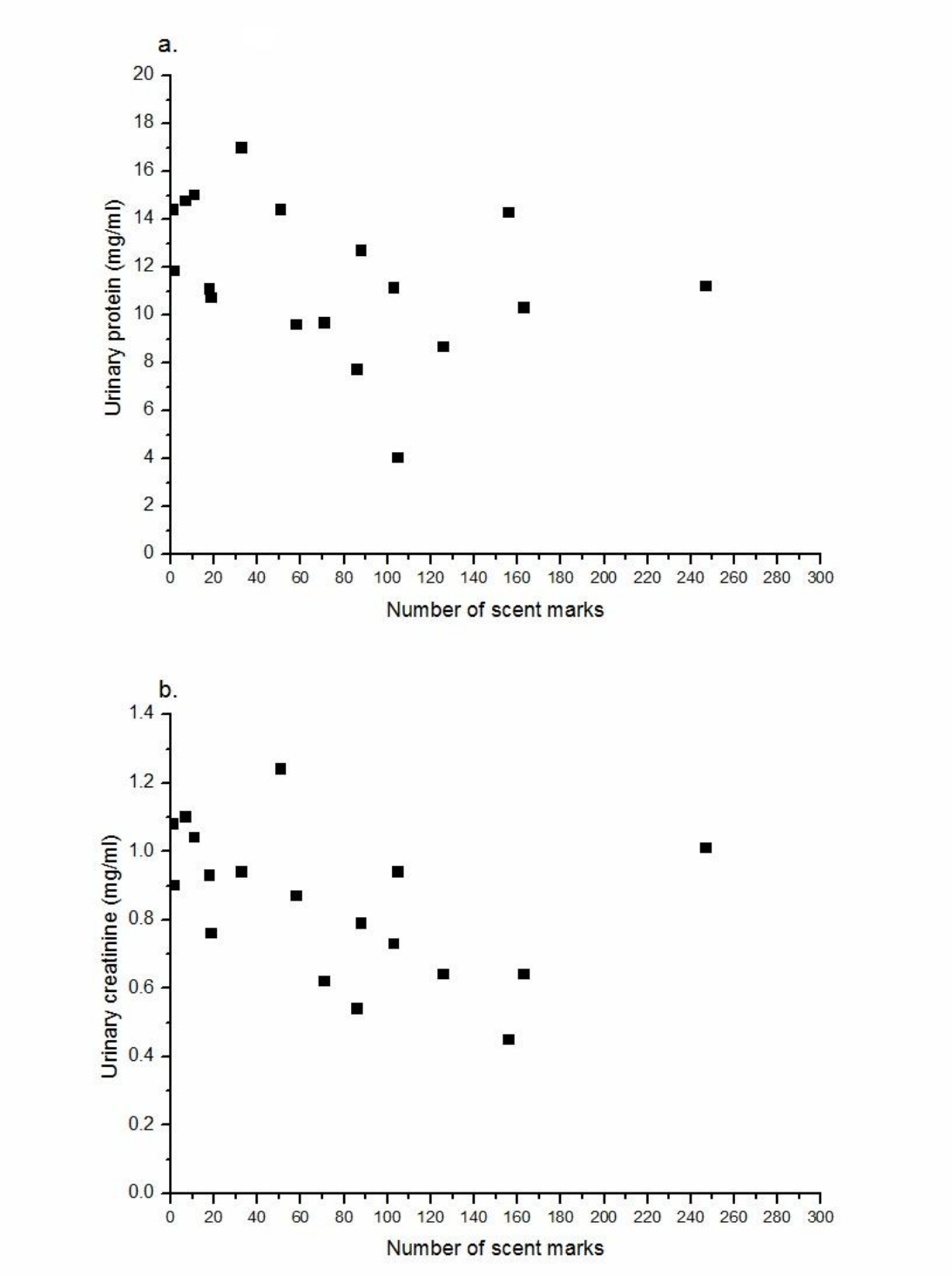


Figure 2.7 Urinary protein and creatinine concentrations and scent mark investment during 'barrier' trials.

Male scent marking was (a) negatively correlated with urinary protein concentration *(*r_s = -0.48, p = 0.046 and (b) negatively correlated with creatinine concentration (r_s = -0.53, p = 0.03). Males that deposited a relatively high number of scent marks had very dilute urine containing a low concentration of protein.

During these trials the amount of time females and males spent sniffing, chewing and pulling at the mesh section of the barrier was recorded as an indicator of interest and attraction; this was positively correlated with male investment in ultrasonic calls (female: Spearman's rho; $r_s = 0.48$, $p = 0.04$; male: Spearman's rho; $r_s = 0.49$, $p = 0.04$; **Figure 2.8**).

In addition, male and female behaviours were recorded during interactions; primary patterns of behaviour were obtained via principal components analysis. The three components from the PCA on male behaviour with an Eigenvalue > 1 are shown in **Table 2.2**. The first component accounted for 45% of variance in the data and contrasted affiliative behaviours and mount latency with the number of mounting attempts. This component was positively correlated with male calling investment (Spearman's rho; $r_s = 0.62$, $p = 0.008$; **Figure 2.9a**), but negatively correlated with scent marking (Spearman's rho; $r_s = -0.50$, $p = 0.04$; **Figure 2.9b**). Males that displayed a high level of affiliative behaviour called at a high rate, deposited relatively few scent marks and made few mounting attempts. The second component accounted for 19% of variance in the data and contrasted following the female with grooming the female; the third component accounted for 18% of variance of the data and contrasted sniffing female genitals and mounting attempts with mount latency. Neither of these components correlated with male signalling investment.

The principal components analysis for female behaviour revealed two components with an Eigenvalue > 1; these are shown in **Table 2.3**. The first component accounted for 45% of variance in the data. This component contrasted sniffing and grooming the male with rejection of mounting attempts and was negatively correlated with the number of scent marks deposited by males (Spearman's rho: $r_s = -0.66$, $p = 0.004$; **Figure 2.10**). This component was also correlated with the first component obtained from the PCA on male behaviour (Spearman's rho: $r_s = 0.49$, $p = 0.048$; **Figure 2.11**). Females therefore sniffed and groomed males more when relatively few scent marks were deposited and males displayed a high level of affiliative behaviour. The second PCA component accounted for 39% of variance, contrasting following the male and sniffing male genitals with rejection of mounting attempts; this component was not correlated with male signalling investment. Despite comprising behaviours similar to those in PCA component 3 for male behaviour, the two were not correlated (Spearman's rho: $r_s = 0.02$, $p = 0.94$).

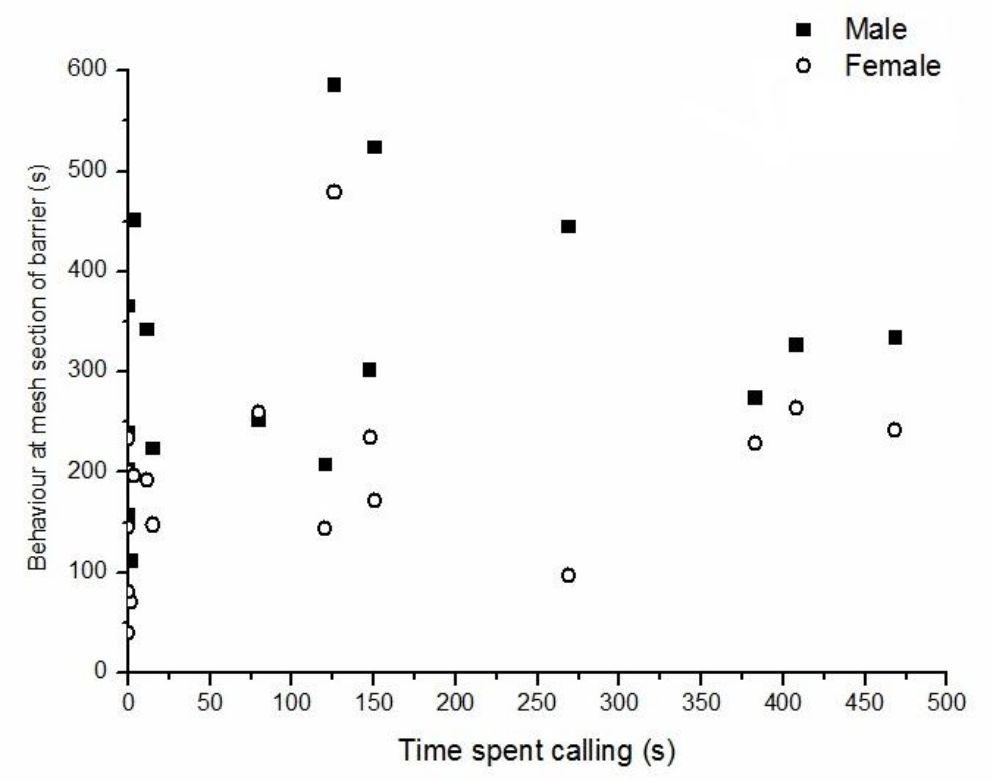


Figure 2.8 Male calling and behaviour at the mesh partition of barrier.
Male (black squares) and female (open circles) behaviour was positively correlated with male calling investment when individuals were separated by a barrier (Male: $r_s = 0.49$, $p = 0.04$; female: $r_s = 0.48$, $p = 0.04$). Behaviours included in this analysis were sniffing, chewing and pulling on the mesh area of the divider.

	PC1	PC2	PC3
Sniff female genitals	0.55	0.01	0.76
Sniff female face	0.69	0.43	-0.18
Groom female	0.71	-0.48	0.33
Follow female	0.39	0.83	0.12
Mount latency	0.89	-0.21	-0.21
Mounting attempts	-0.71	0.15	0.55
Variance	45%	19%	18%

Table 2.2 Principal component weights describing male behaviour exhibited during interactions.
Component 1 contrasted affiliative behaviours and mount latency with the number of mounting attempts, component 2 contrasted sniffing and following with grooming, and component 3 contrasted sniffing genitals and mounting attempts with mount latency.

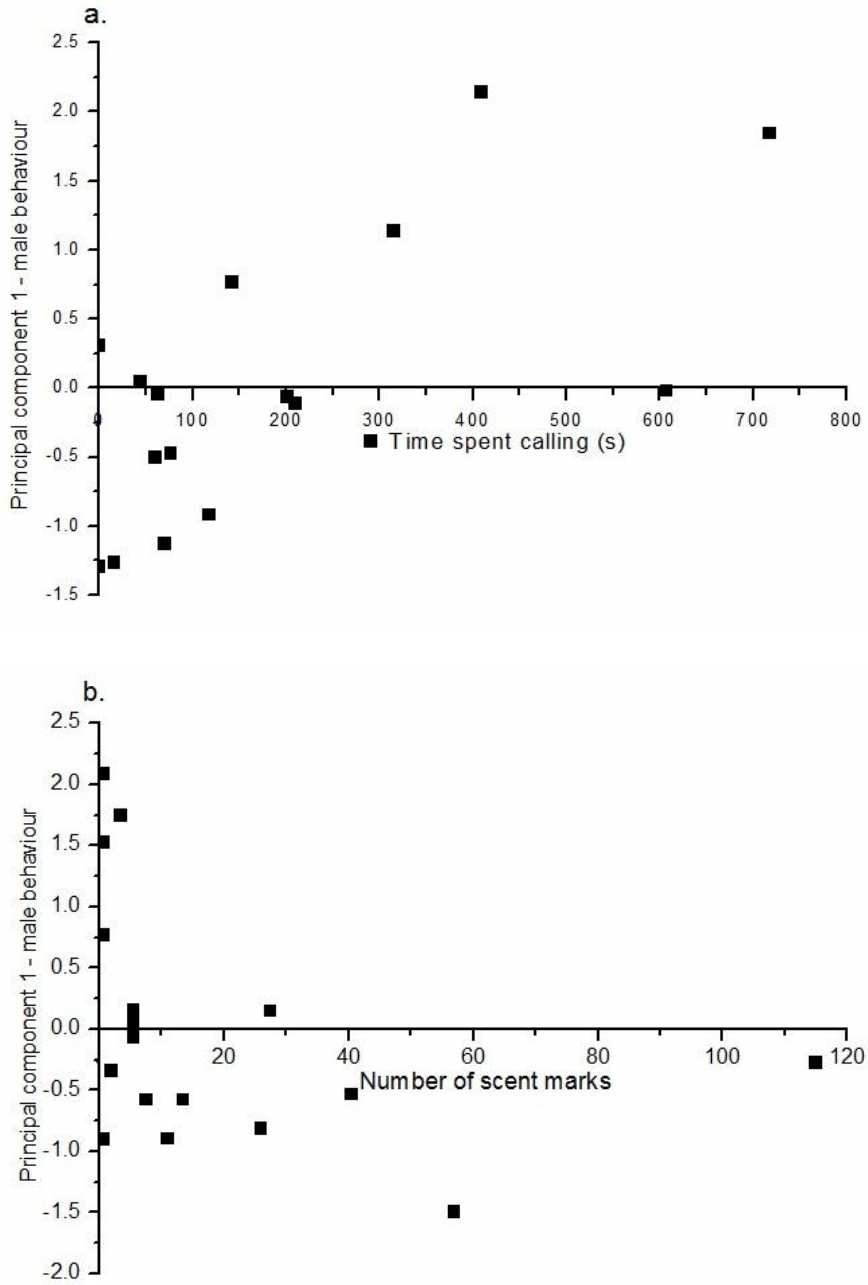


Figure 2.9 Male signalling and behaviour during interactions.
The main patterns of male behaviour were obtained from a principal components analysis as shown in **Table 2.1**; individual scores for principal component 1 contrasting affiliative behaviour and mount latency with mounting attempts were positively correlated with (a) male calling and (b) scent marking (calling: $r_s = 0.62$, $p = 0.008$; scent marking: $r_s = -0.50$, $p = 0.04$). Males that invested heavily in calling typically displayed a high level of affiliative behaviour towards females but also tended to make fewer mounting attempts. Males that invested heavily in scent marking typically spent less time grooming and sniffing the female but made a high number of mounting attempts.

	PC1	PC2
Sniff male genitals	-0.24	0.89
Sniff male face	0.96	0.01
Groom male	0.96	0.05
Follow male	-0.22	0.89
Flee mounting	-0.55	-0.63
Variance	45%	39%

Table 2.3 Principal component weights describing female sexual behaviour during interactions.
Component 1 contrasted sniffing and grooming with fleeing from mounting attempts and component 2 contrasted sniffing and following with fleeing from mounting attempts.

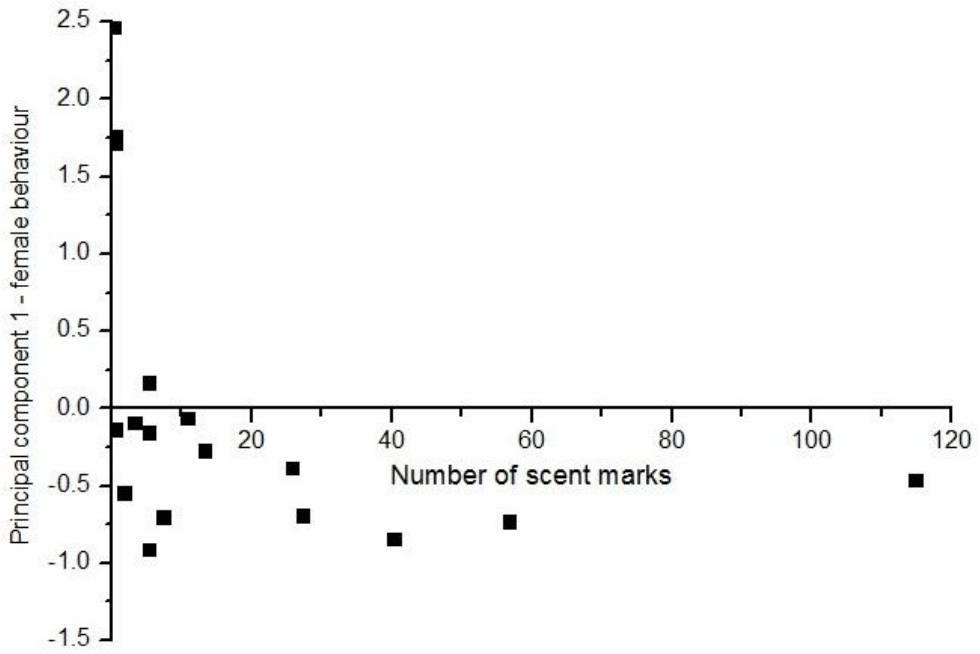


Figure 2.10 Male scent mark investment and female behaviour.
The main patterns of female behaviour were obtained from a principal components analysis as shown in **Table 2.3**. Principal component 1, contrasting sniffing and grooming the male with rejection of mounting attempts, was negatively correlated with male scent marking (r_s = -0.66, p = 0.004). Females exhibited a high level of affiliative behaviour when males typically produced low numbers of scent marks.

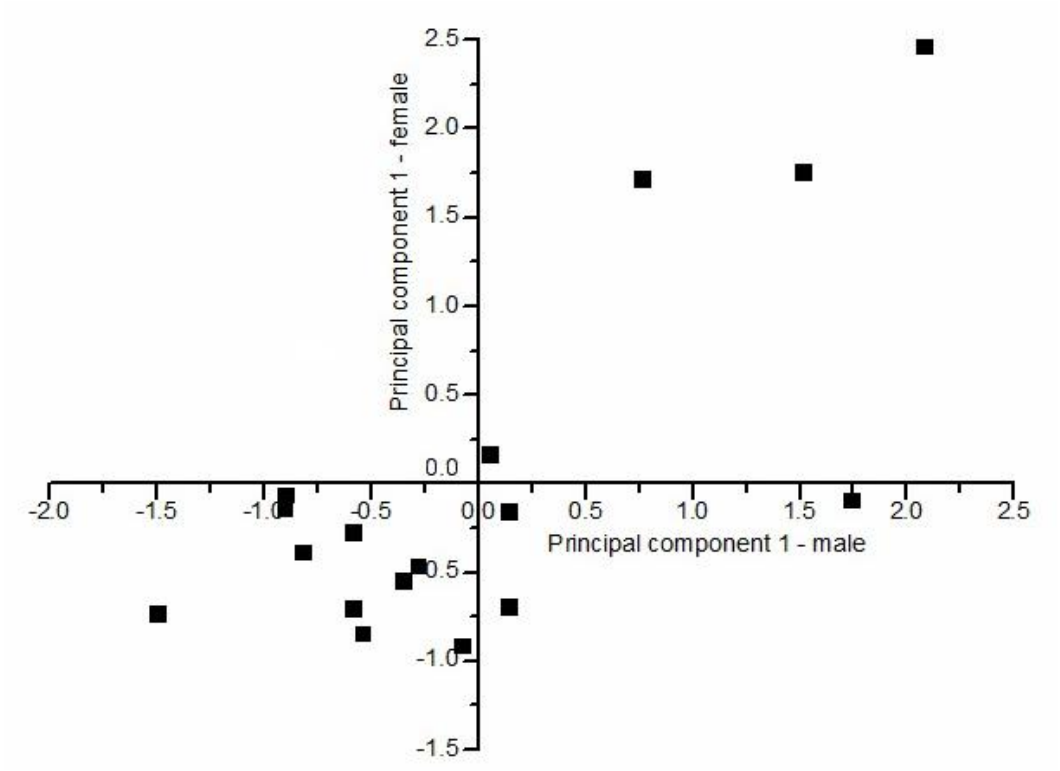


Figure 2.11 Male and female behaviour during interactions.
The main patterns of behaviour were derived from separate principal components analyses on female and male behaviour during interactions; the components obtained are shown in **Tables 2.2** and **2.3**. Individual scores for principal component 1 describing male behaviour were correlated with individual scores for principal component 1 describing female behaviour ($r_s = 0.48$, $p = 0.048$). Males displaying a high level of affiliative behaviour interacted with females that spent the greatest amount of time sniffing and grooming the male.

EXPERIMENT 2

The second experiment was conducted to extend the findings of interaction trials in the first experiment and to investigate other factors that may be linked to male signalling investment. As a result of single sexual interactions in experiment one, only correlations between male signalling investment and the behaviour of males and females could be identified. In this second experiment, subjects participated in multiple sexual interactions to establish causality and assess whether male investment in signalling is a stable trait or influenced by the behaviour of different individual females. As in experiment one, in addition to measuring the number of scent marks and call rate, during interactions the total duration of expression of affiliative behaviours (e.g. sniffing and following the other animal) and male mounting activity was recorded. Additionally, the links between male signalling investment, corticosterone and male reproductive physiology were assessed.

2.4 METHODS

2.4.1 Subject and stimulus animals

Subject males (n = 18) were CD-1 laboratory mice bred in house (parents obtained from Harlan UK) and aged 12 months at the start of the experiment. 18 adult female C57BL/6JOlaHsd laboratory mice bred in house (parents obtained from Harlan UK) and aged 12 months acted as stimulus animals. Males were housed singly in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK) and females were housed in same-sex pairs in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK); pairs were maintained throughout the study. Mice in this experiment were different to individuals used in the first experiment and had no prior sexual experience but had previously been exposed to conspecific odours. Soiled male nesting material was added to the home cages of females three days prior to each interaction to induce oestrus (Cheetham *et al.,* 2007). Throughout the experiment all animals were maintained on a reversed 12:12 light cycle (lights off at 08:00h) and on Corn Cob Absorb 10/14 substrate with paper wool bedding material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, MO, USA). Acrylic tunnels (5cm x

15cm) and red plastic houses (Techniplast, NJ, USA) were provided for home cage enrichment.

2.4.2 Experimental procedure

Male subjects and female stimulus animals participated in three interactions, each with a different individual of the opposite sex. All trials were separated by between 5 and 7 days. All interactions took place during the dark phase of the light cycle, under red lighting at a consistent level of illumination and location, and in clean test arenas measuring 45cm x 28cm x 13cm (MB1, North Kent Plastics Ltd., UK). Animals were all handled and transferred between test arenas and home cages using clear acyclic tunnels to minimise stress and anxiety during testing (Hurst and West, 2010). Interactions lasted 30 minutes and took place in a room separate from the rest of the colony. Benchkote paper (Fisher Scientific, Nottingham, UK) was placed on the base of each test arena to assist in the recording of scent marks.

2.4.3 Scent mark analysis and recording ultrasonic calls

Both scent marks and ultrasonic calls were recorded and analysed using the same methods as in experiment one. Males were then given a rank according to their scent marking and calling relative to other males during each interaction. For example, a male assigned rank 1 spent the longest amount of time calling or produced the greatest number of scent marks. Conversely, a male assigned rank 18 produced the fewest calls or scent marks. Each male was therefore assigned six ranks; one for each of the two signal types in each of three interactions. Mean scent marking and calling investment made by each male across the three interactions was also calculated.

2.4.4 Urine and faecal sample collection

Urine samples were collected from male subjects 2h before each interaction to provide samples for analysis of baseline levels of testosterone, protein and creatinine as described in section 2.2.4 of experiment one. Additionally, urine was collected 48 hours

after each interaction so changes in urinary hormones and protein could be measured. Faecal samples were collected before interactions for analysis of corticosterone and 4h after each interaction to maximise the chances of recording corticosterone at peak levels as a result of the interaction (Touma *et al.*, 2003). All samples were collected by confining males above a cage on a mesh grid for a maximum of 2h. Cages under males were checked every 30 minutes and if urine or faeces were present they were collected immediately and stored at -20°C.

2.4.5 Measurement of urinary and faecal hormones

The methods used for measurement of testosterone, protein and creatinine concentration were the same as described in sections 2.2.5 and 2.2.6 of experiment one. Faecal corticosterone was measured by wet weight shaking extraction followed by an enzyme immunoassay; a process validated with the assistance of Amanda Davidson and Katie Edwards (Mammalian Behaviour and Evolution Group, University of Liverpool). Faecal samples were weighed, transferred into extraction vials and 3ml of 90% methanol added. Vials were then spun until contents were well mixed and placed on a rotator to agitate overnight. Samples were then centrifuged for 20 minutes at 1800rpm and the supernatant poured into glass extraction tubes. This was then dried down in a warm water bath under air in a fume hood; the residue was reconstituted with 1ml 100% methanol and stored at -20°C.

Corticosterone was obtained from Sigma chemicals, UK, and antibodies to corticosterone and corresponding horseradish peroxidise conjugates were obtained from the Clinical Endocrinology Laboratory at the University of California, Davis, USA. NUNC Maxisorb plates were coated in 50µl of antibody stock diluted 1:11,000 in a coating buffer (50mmol/l bicarbonate buffer, pH 9.6) then stored for 12-14 hours at 4°C. Wash solution (0.15mol/l NaCl solution containing Tween 20) then rinsed away any unbound antibody. 25µl of phosphate buffer (0.1mol/l sodium phosphate buffer, pH 7.0 containing 8.7g NaCl and 1g BSA), 50µl of standard or sample (urine samples diluted 1:10 in phosphate buffer), then 50µl of corticosterone horseradish peroxidase (diluted 1:65,000) were added to wells. Plates were incubated at room temperature for 2 hours before rewashing. 100µl of substrate solution (Citrate buffer, H_2O_2 and 2,2'-azino-bis) was added and left to incubate at room temperature until the optical density

of blank wells (i.e. those containing 0pg corticosterone standard) reached one. Plates were read with a single filter at 405nm.

2.4.6 Measurement of urinary darcin concentration

Darcin, an involatile sex pheromone, is a protein expressed in the urine of male mice. This protein is particularly important in sexual communication for males when attracting females via olfactory signals. To determine the amount of darcin present in urine collected, samples were analysed using SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) to separate darcin from the rest of the MUPs following methods described by Laemmli (1970) (**Figure 2.12**). Urine samples were diluted to 0.25μg/μl protein with ddH_2O and added in a 1:1 ratio to 2x sample buffer (1M Tris pH6.8, glycerol, 2% SDS, dithiothreitol (DTT)). Samples were centrifuged briefly at 9000rpm, boiled at 100°C for 5 min and then centrifuged a second time. Samples were then loaded onto gels and electrophoresis run at a constant 200V for approximately 45 min. Broad range molecular weight markers (Bio-Rad Laboratories Ltd., Hertfordshire) were also run in a control lane. Protein bands were visualised using by staining gels in 200ml Coomassie Brilliant Blue overnight, followed by destaining in a solution of methanol, acetic acid and ddH_2O (30:5:65 v/v/v) for 2 hours or until the majority of background staining was removed. Densitometry was then performed on all bands by scanning gels and analysing images using Total Lab TL100 software to assess the percentage of darcin (in relation to other MUPs) present in each sample.

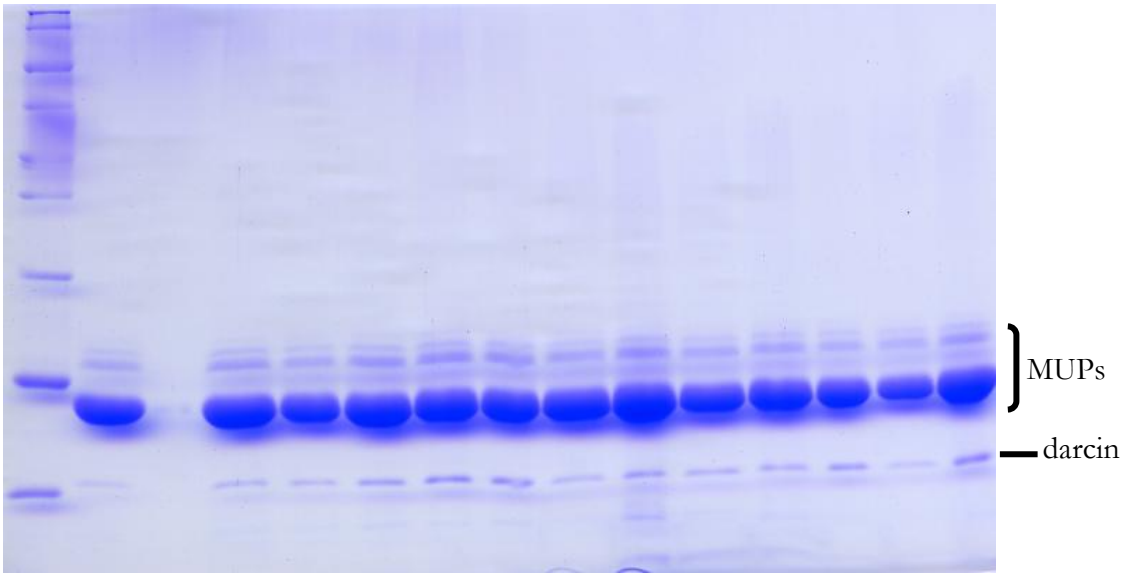


Figure 2.12 Male CD-1 urine samples resolved by 15% SDS-PAGE.

The thicker bands contain the majority of male MUPs; the smaller band further down the gel indicates the male specific MUP, darcin.

2.4.7 Analysis of behaviour during interactions

All trials were recorded onto DVD and decoded using the same methods described in section 2.2.8 of experiment one. Female behaviours recorded were: total duration of sniffing male facial area, sniffing male genitals, grooming the male and following the male and the number of times females fled from mounting attempts. Male behaviours recorded were: total duration of sniffing female facial area, sniffing genitals of the female, grooming the female and following closely behind the female, as well as latency to first mounting attempt and number of mounting attempts.

2.4.8 Analysis of sperm count and reproductive physiology

The body mass of males was recorded at the end of the experiment. Males were then humanely culled. The wet mass of the testes, seminal vesicles and the preputial gland were recorded. The concentration of sperm in the caput of the left epididymis was also estimated following methods previously described by Ramm and Stockley (2009). The caput was dissected from the body and the tissue added to 0.1ml of 1% citrate solution in a Petri dish. This tissue was macerated with a scalpel blade and a further 0.9ml of citrate solution added. This mixture was allowed to stand for no more than 2 minutes before 10µl was transferred to each chamber of an improved Neubauer haemocytometer. Once prepared, the haemocytometer was left to stand for 15 minutes in a sealed, moist container before sperm were counted manually under a microscope. Counts were duplicated for each chamber and conducted blind relative to subject identity.

2.4.9 Data analysis

All statistical tests were carried out using the SPSS software package, version 20. Scent marking and calling data could not be normalised (assessed by Kolmogorov Smirnov and Shapiro Wilks tests $p < 0.05$), so Spearman's rank correlations with untransformed data tested for relationships between signalling investment, urinary hormone and protein levels, faecal hormone levels and patterns of sexual behaviour. Sexual behaviour was analysed using a separate principal components analysis for

female and male behaviours. Only components with an Eigenvalue of one or greater were used in subsequent analyses. Where hormone concentration and male signalling investment was compared across interactions, non-parametric Friedman and Wilcoxon signed-ranks tests were used due to the non-normal distribution of data; comparisons of behaviour across interactions was conducted using repeated measures ANOVAs.

2.5 RESULTS

2.5.1 Is male signalling investment or behaviour of males and females consistent across multiple interactions?

The scent marking and calling of individual males was recorded during three interactions with different females. Among individuals the number of scent marks produced ranged from 0-191 in interaction 1, 0-308 in interaction 2 and 0-264 in interaction 3 (**Figure 2.13a-c**), showing a high degree of inter-individual variation in scent mark investment. The median number of marks produced during interactions 1, 2 and 3 was 5, 16 and 35 respectively, suggesting the majority of males produced very few marks. The time males spent calling also varied among individuals, ranging from 0-623s in interaction 1, 19-720s in interaction 2 and 41-701s in interaction 3 (**Figure 2.13d-f**); the median time spent calling in interactions 1, 2 and 3 was 138s, 322s and 340s respectively. During the first interaction most males spent a relatively low amount of time calling and only a small number of individuals called at the highest rates. However, during the second and third interactions, variation among males was not as heavily skewed. When signalling investment was compared across multiple interactions, scent marking did not differ significantly (Friedman test: χ^2 (2) = 4.52, p = 0.11; **Figure 2.14a**), suggesting investment in this signal remains stable. However, time spent calling did differ significantly across the three sexual interactions (Friedman test: χ^2 (2) = 11.44, p = 0.003; **Figure 2.14b**); male calling investment increased over the course of the experiment.

To further understand the consistency of male signalling investment, assessment of male signalling relative to other individuals was carried out by assigning each male a rank according to investment during each interaction (rank 1 for greatest investment, rank 18 for the lowest). This controls for any change in behaviour across multiple interactions. There was a significant correlation between the ranks of individual males across the three interactions for both scent marking (ranks for interactions 1 and 2: Spearman's rho: r_s = 0.65, p = 0.003; ranks for interactions 2 and 3: Spearman's rho r_s = 0.64, p = 0.004; **Figure 2.15a**) and calling (ranks for interactions 1 and 2: Spearman's rho: r_s = 0.66, p = 0.003; ranks for interactions 2 and 3: Spearman's rho r_s = 0.77, p < 0.001; **Figure 2.15b**). Males that invested relatively heavily in calling or scent marking during the first interaction invested similarly during subsequent interactions.

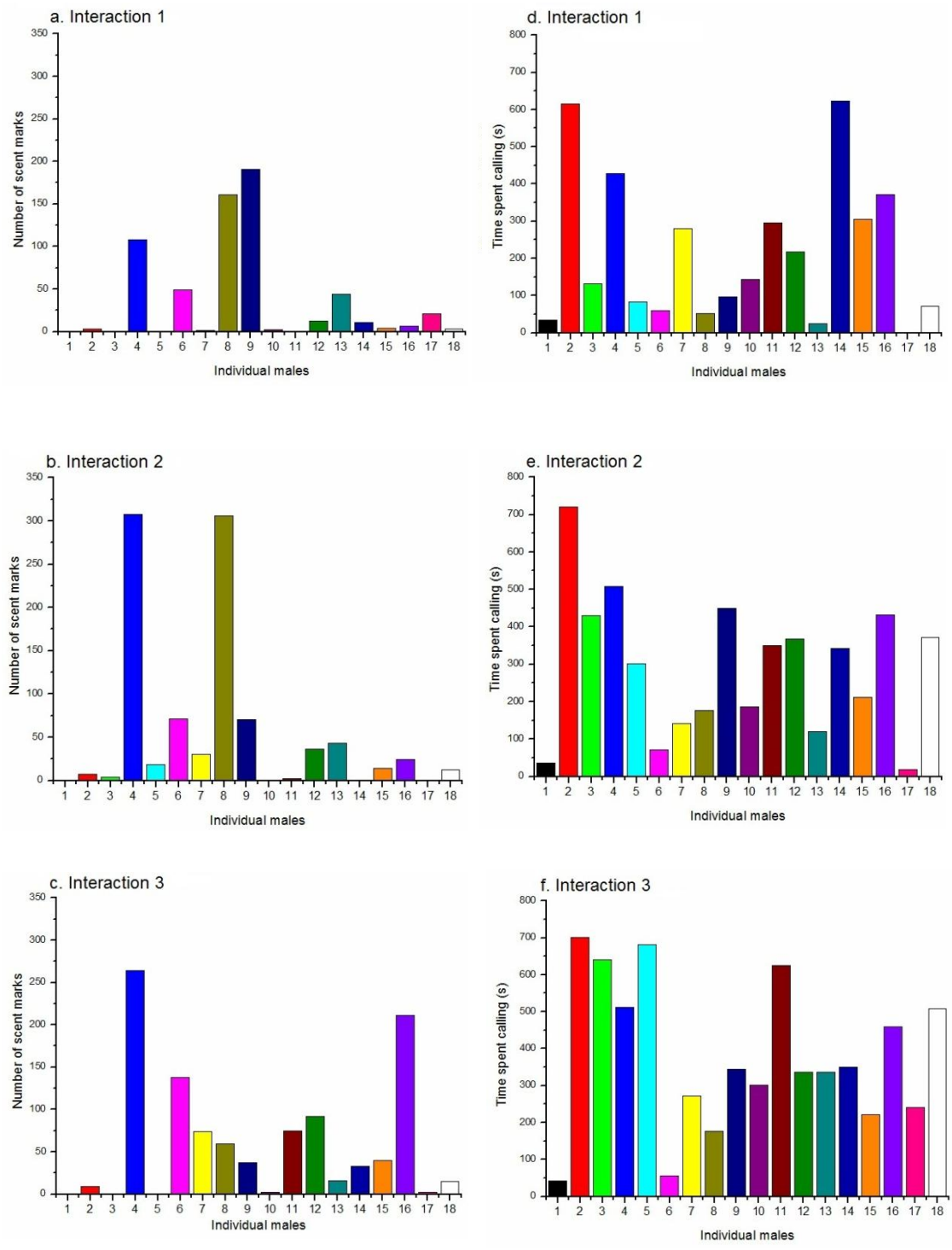


Figure 2.13 Male signalling during three sexual interactions.
Each coloured bar represents a specific, individual male. Colours are consistent across panels (i.e. the red bar represents the same individual male in all six panels); colours are also consistent with those in **Figure 2.15**. The number of scent marks deposited ranged from 0-191, 0-308 and 0-264 in interactions 1, 2 and 3 respectively (panels a, b and c). The time males spent calling ranged from 0-623s, 19-720s and 41-701s in interactions 1, 2 and 3 respectively (panels d, e and f).

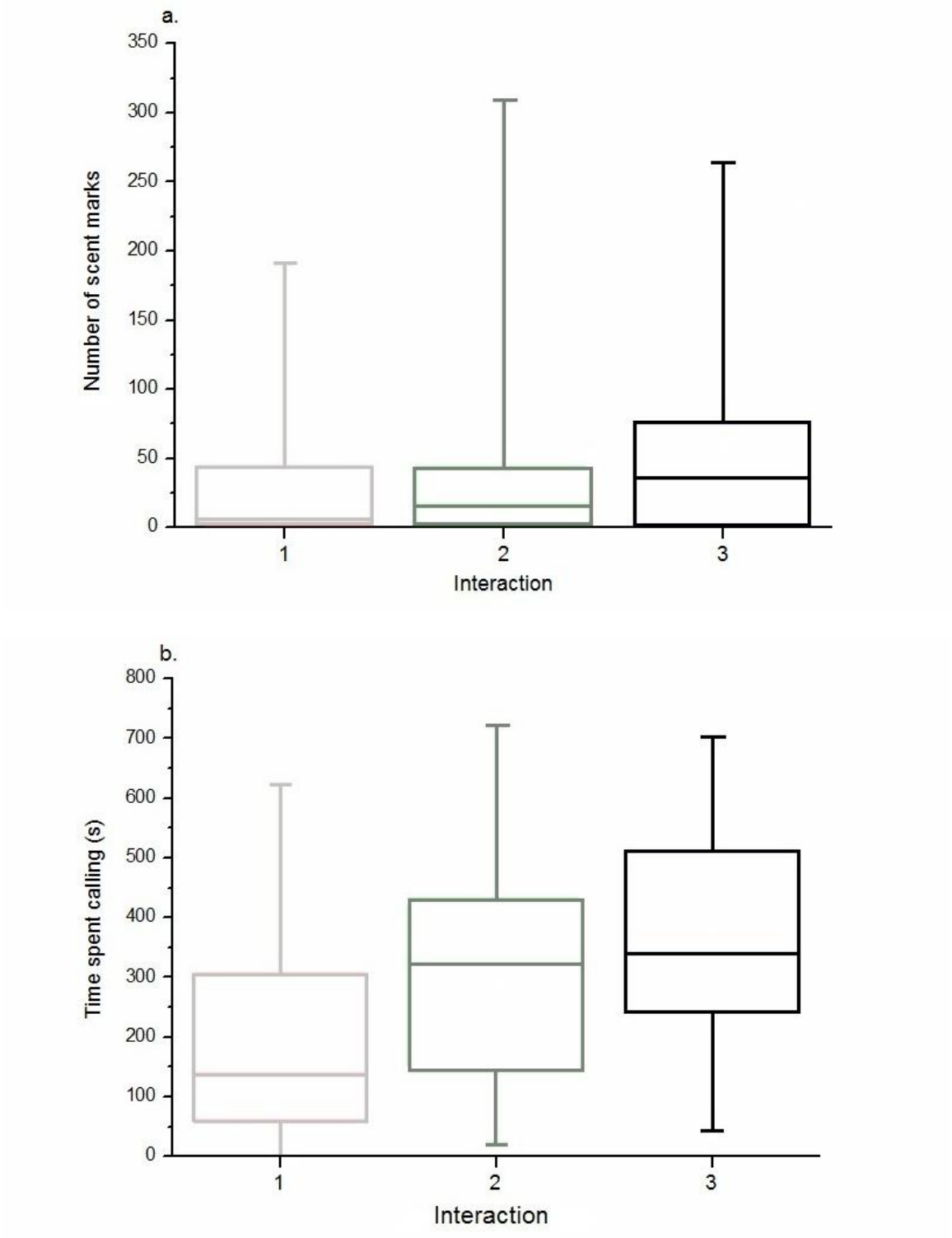


Figure 2.14 Male signalling across three sexual interactions.
Box plots showing male (a) scent marking and (b) calling during three interactions with females; scent marking did not differ significantly across interactions but calling investment increased (scent marking: χ^2 (2) = 4.52, p = 0.11; calling: χ^2 (2) = 11.44, p = 0.003).

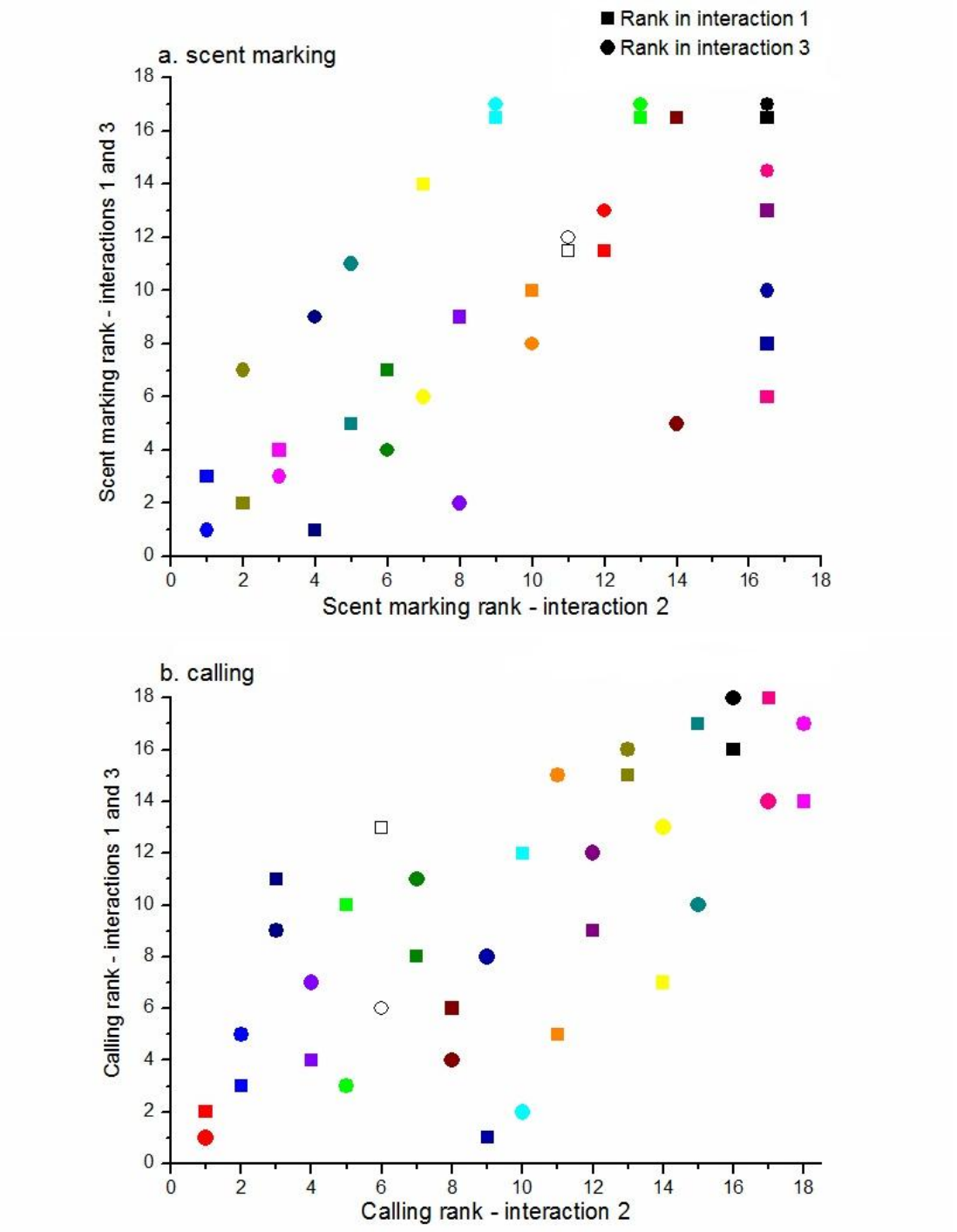


Figure 2.15 Ranks of individual males for signal investment during interactions. Each colour represents the rank of a specific individual male based on the signalling investment of that male as shown in **Figure 2.13**; colours representing individuals are consistent across the two figures. Individual ranks for scent marking and calling were positively correlated across the three interactions; males ranked highly in investment during one interaction typically had the same or similar rank in subsequent interactions (**scent marking**: interactions 1 and 2: $r_s = 0.65$, $p = 0.003$; interactions 2 and 3: $r_s = 0.64$, $p = 0.004$; **calling**: interactions 1 and 2: $r_s = 0.66$, $p = 0.003$; interactions 2 and 3: $r_s = 0.77$, $p < 0.001$).

Using the same statistical methods applied to data in experiment one, the primary patterns of behaviour during interactions were obtained via principal components analysis. In contrast to behavioural data from experiment one, only two components from the PCA on male behaviour had an Eigenvalue > 1; these are shown in **Table 2.4**. The first component accounted for 33% of variance in the data and as in experiment one contrasted affiliative behaviours and mount latency with the number of mounting attempts made. Individual scores for this component differed significantly across the three interactions (repeated measures ANOVA: $F_{(2,34)} = 9.52$, $p = 0.001$; **Figure 2.16a**); male affiliative behaviour declined during the experiment but sexual motivation may have increased as the number of mounting attempts increased and mount latency decreased. The second component accounted for 29% of the variance in the data and contrasted sniffing and mounting attempts with mount latency. Individual scores for this component did not differ significantly across the three interactions in this experiment (repeated measures ANOVA: $F_{(2,34)} = 0.12$, $p = 0.88$; **Figure 2.16b**).

Two components from the PCA on female behaviour had an Eigenvalue > 1; these are shown in **Table 2.5**. The first component accounted for 39% of variance in the data. This component contrasted affiliative behaviour (sniffing, grooming, following) with the number of times females rejected mounting attempts. Mean scores for this component were significantly different across the three interactions (repeated measures ANOVA: $F_{(2,34)} = 4.34$, $p = 0.02$; **Figure 2.17a**). Additionally, in contrast to the first experiment, component 1 for female behaviour was not correlated with component 1 for male behaviour in any of the three sexual interactions (interaction 1: Spearman's rho: $r_s = 0.15$, $p = 0.54$; interaction 2: Spearman's rho: $r_s = 0.05$, $p = 0.85$; interaction 3: Spearman's rho: $r_s = 0.35$, $p = 0.16$). This suggests the level of affiliative behaviour and interest of females was not linked to the same behaviours in males. The second component accounted for 24% of variance in the data and contrasted grooming and sniffing the facial area of males with following and sniffing males' genitals. Mean scores for this component did not differ significantly across the three interactions of the experiment (repeated measures ANOVA: $F_{(2,34)} = 0.92$, $p = 0.41$; **Figure 2.17b**).

	PC1	PC2
Sniff female genitals	0.18	0.79
Sniff female face	0.50	0.71
Groom female	0.68	0.21
Follow female	0.57	0.13
Mount latency	0.73	-0.49
Mounting attempts	-0.61	0.58
Variance	33%	29%

Table 2.4 Principal component weights describing male behaviour during interactions.
Component 1 contrasted affiliative behaviours and mount latency with the number of mounting attempts and component 2 contrasted sniffing with mount latency.

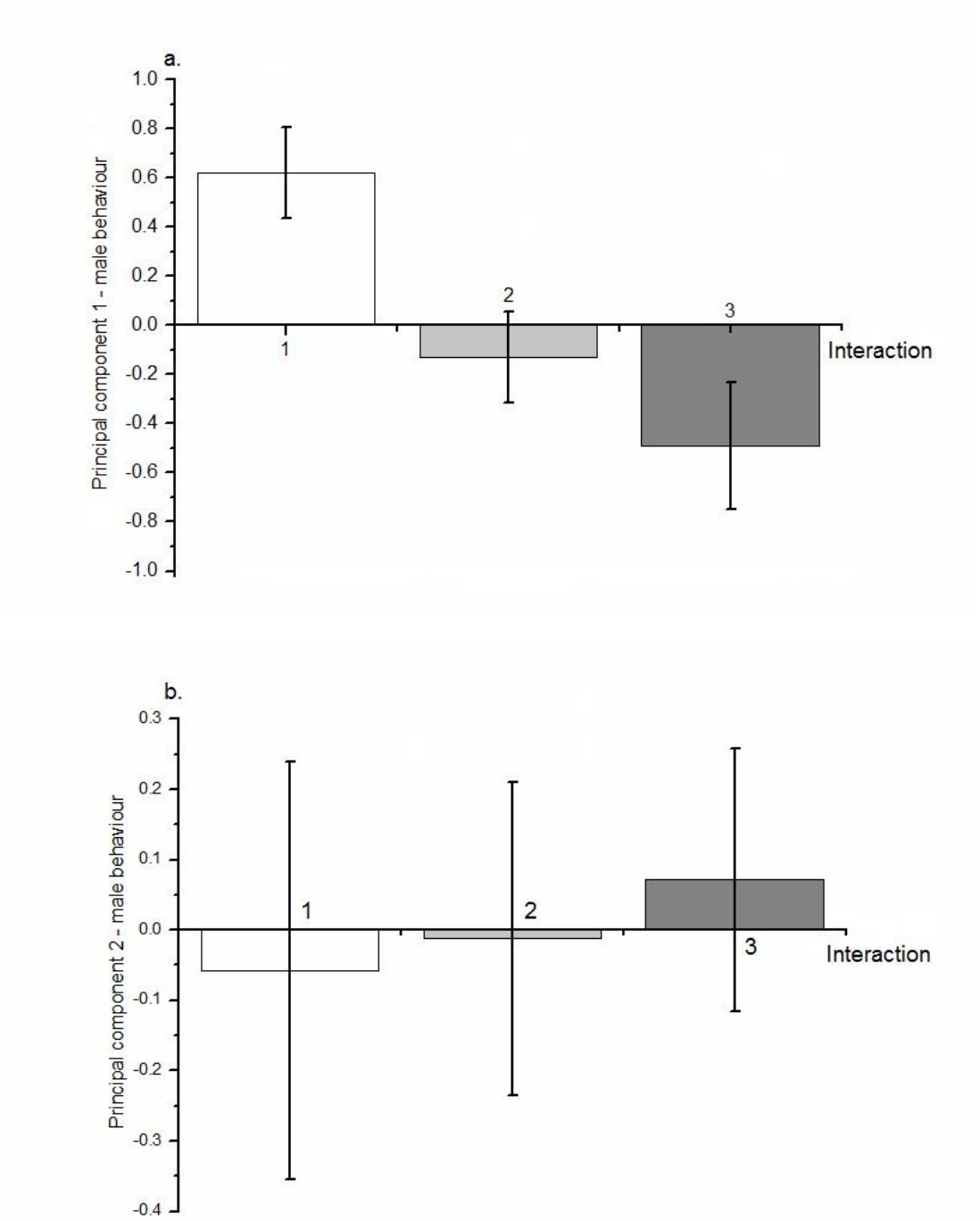


Figure 2.16 Behaviour of males during three sexual interactions.
Mean scores for (a) principal component 1 contrasting affiliative behaviour and mount latency with mounting attempts differed significantly across the three interactions ($F_{(2,34)}$ = 9.52, p = 0.001). Scores for (b) principal component 2 contrasting sniffing with mount latency did not differ significantly ($F_{(2,34)}$ = 0.12, p = 0.88). Bars represent means + SEM.

	PC1	PC2
Sniff male genitals	0.64	-0.47
Sniff male face	0.72	0.51
Groom male	0.64	0.55
Follow male	0.58	-0.64
Flee mounting attempts	-0.52	0.09
Variance	39%	24%

Table 2.5 Principal component weights describing female behaviour during interactions.
Component 1 contrasted affiliative behaviours with fleeing from mounting attempts and component 2 contrasted facial sniffing and grooming with following and genital sniffing.

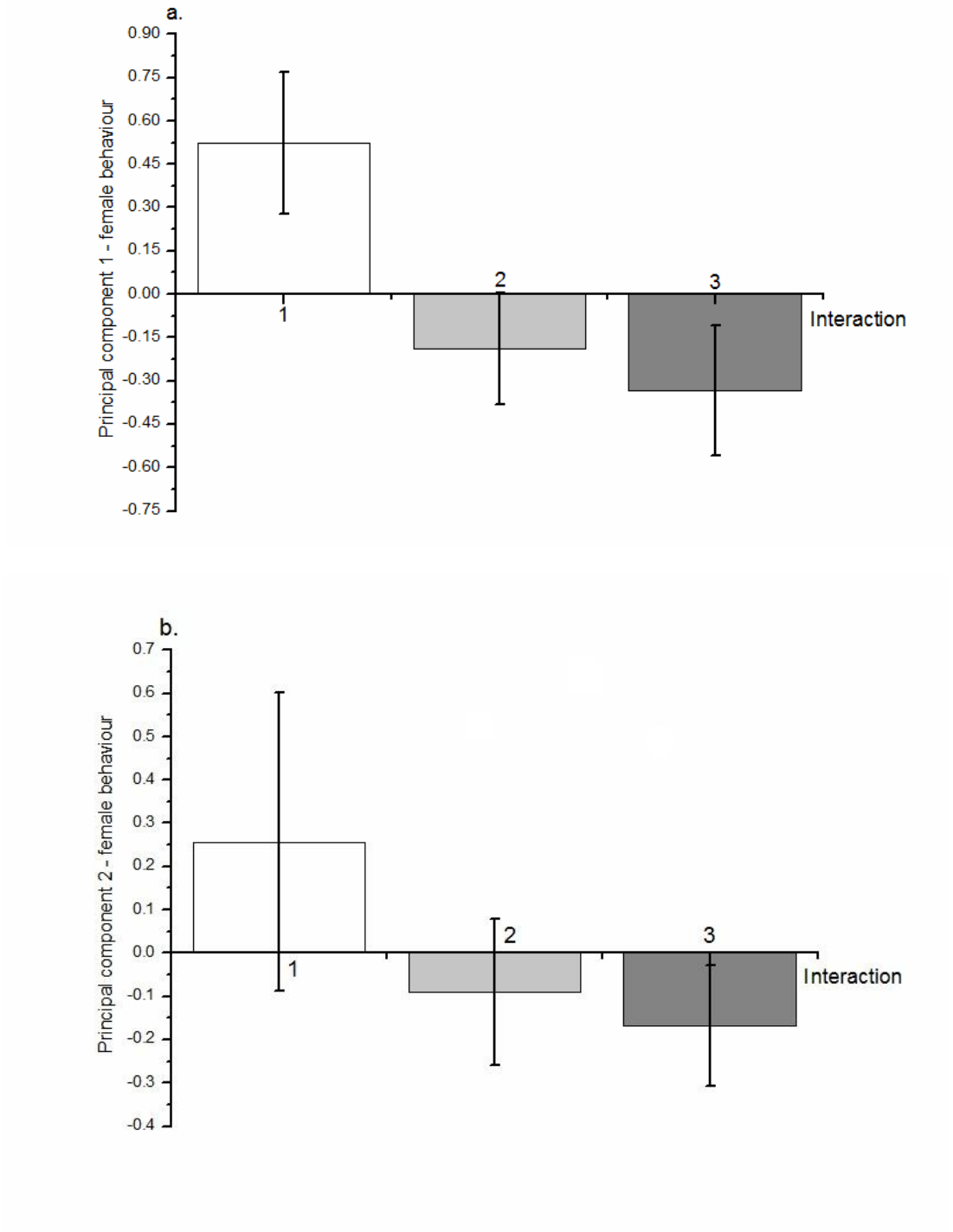


Figure 2.17 Behaviour of females during three sexual interactions.
Mean scores for (a) principal component 1 contrasting affiliative behaviour with fleeing from mounting attempts differed significantly across the three interactions ($F_{(2,34)}$ = 4.34, p = 0.02). Scores for (b) principal component 2 contrasting facial sniffing and grooming with genital sniffing did not differ significantly ($F_{(2,34)}$ = 0.92, p = 0.41). Bars represent means + SEM.

2.5.2 Is male calling investment a reliable indicator of sperm count or reproductive physiology?

Male ultrasound elicitation is restricted to encounters with females and female odours and has been suggested to serve a specific function in courtship and kin recognition (Nyby *et al.*, 1977; Musolf *et al.,* 2010). Calls elicit female approach and females are more likely to associate with a vocalising male over an individual that fails to emit ultrasounds (Pomerantz *et al.*, 1983; Hammerschmidt *et al.,* 2009), but whether calls reliably inform females of any aspects of male quality is unclear. In this experiment, the mean time spent calling was positively correlated with the total sperm count of individuals (Spearman's rho: $r_s = 0.47$, $p = 0.049$; **Figure 2.18**); males investing heavily in calling during interactions had relatively high sperm counts.

2.5.3 Is male investment in specific major urinary proteins linked to accessory gland size?

Secretions from the male preputial gland are added to urine during scent marking and the pheromonal components of these secretions are attractive to females (Bronson and Caroom, 1971). Dominant males have larger preputial glands and these individuals typically scent mark at a higher rate (Desjardins et al., 1973; Harvey *et al.,* 1989). In addition, the seminal vesicles are accessory glands important in the production of seminal fluid proteins such as those involved in copulatory plug formation (Bradshaw and Wolfe, 1977). The production of these proteins as well as MUPs in scent marks may represent a high energetic cost to males (Gosling *et al.,* 2000). As a result, males may need to balance the costs associated with investment in the production of specific MUPs and accessory gland proteins. Seminal vesicle weight was negatively correlated with the mean percentage of darcin relative to other MUPs (Spearman's rho: $r_s = -0.71$, $p < 0.001$; **Figure 2.19**), but not with the total concentration of darcin in urine (Spearman's rho: $r_s = -0.36$, $p = 0.14$). The correlation with darcin percentage remained significant even when seminal vesicle weight was adjusted for the body mass of individual males ($r_s = -0.69$, $p < 0.001$). Males with heavier accessory glands had a small percentage of darcin relative to overall MUP expression (**Table 2.6**). Interestingly, male preputial gland size (absolute or adjusted for body mass) was not correlated with male urinary protein or darcin concentration (**Table 2.7**).

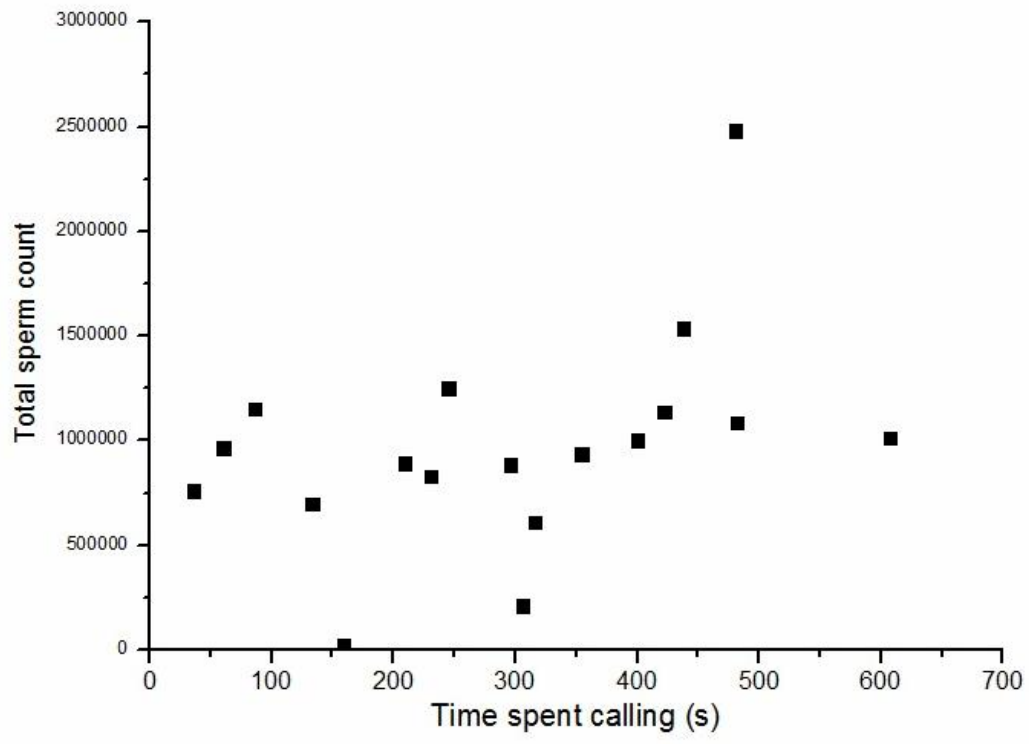


Figure 2.18 Male calling investment and individual total sperm count.
Male calling was positively correlated with sperm count; males that spent more time calling during interactions typically had a higher total sperm count ($r_s = 0.47$, $p = 0.049$).

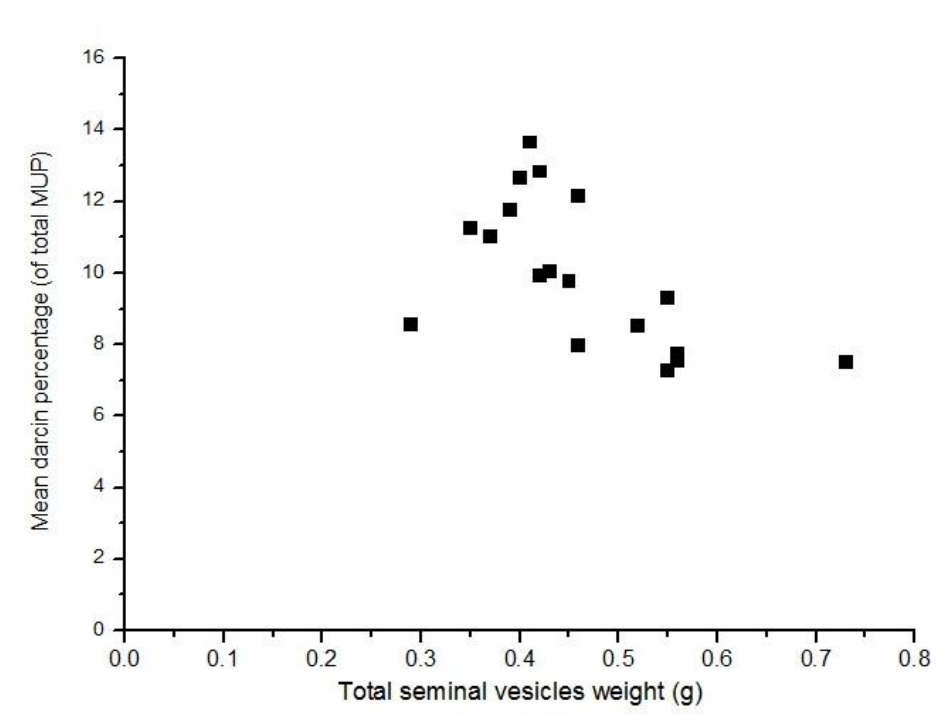


Figure 2.19 Male seminal vesicle weights and urinary darcin percentage (relative to other MUPs).

The combined weight of the male seminal vesicles was negatively correlated with the percentage of MUP expressed as darcin in urine samples collected before interactions (percentage darcin: $r_s = -0.71$, $p < 0.001$). Males with heavy seminal vesicles expressed a low percentage of darcin relative to other males.

	Urinary measure	Spearman's rho, r_s	P value
Absolute seminal vesciles weight	Protein concentration	0.16	0.53
	Darcin concentration	-0.37	0.14
	Darcin percentage	**-0.71**	**<0.001*** **
Adjusted seminal vesicles weight	Protein concentration	0.001	0.99
	Darcin concentration	**-0.47**	**0.047***
	Darcin percentage	**-0.69**	**<0.001*** **

Table 2.6 Analysis of links between male seminal vesicle size and urinary protein and darcin expression.

Male seminal vesicle size (absolute or adjusted for body mass) was correlated with the percentage of darcin relative to other MUPs. Urinary darcin and protein concentrations used in these analyses were adjusted for urine dilution. * $p < 0.05$; *** $p < 0.001$

	Urinary measure	Spearman's rho, r_s	P value
Absolute preputial gland weight	Protein concentration	-0.09	0.72
	Darcin concentration	0.06	0.81
	Darcin percentage	0.21	0.42
Adjusted preputial gland weight	Protein concentration	-0.15	0.54
	Darcin concentration	0.07	0.79
	Darcin percentage	0.23	0.35

Table 2.7 Analysis of links between male preputial gland size and urinary protein and darcin expression.

Male preputial gland size (absolute or adjusted for body mass) was not correlated with any measures of urinary protein or darcin concentration. Urinary darcin and protein concentrations used in these analyses were adjusted for urine dilution.

2.5.4 Is male signalling or behaviour linked to individual levels of the stress hormone corticosterone?

Corticosterone is a major steroid hormone produced during the stress response. In some rodents, an increase in stress due to social defeat or manipulations such as foot shock and immobilisation is linked with a reduction in sexual activity, motivation and signalling (Retana-Marquez *et al.,* 1996; Lumley *et al.,* 1999). Male corticosterone concentration in faecal samples collected before and after each interaction was therefore analysed to determine whether levels of this hormone were linked with individual behaviour or signalling investment. Individual corticosterone was not correlated with any measures of sexual behaviour or signalling investment. In addition, when comparing the corticosterone concentration of samples collected before and after each interaction, no significant changes were detected (Wilcoxon tests: Interaction 1: $Z = -1.91$, $p = 0.06$; Interaction 2: $Z = 0.86$, $p = 0.42$; Interaction 3: $Z = 0.98$, $p = 0.35$; **Figure 2.20**). However, there was a significant difference in the corticosterone present in samples collected before each of the three interactions (Friedman test: $\chi^2(2) = 8.00$, $p = 0.02$); male corticosterone levels prior to the third interaction were significantly greater than levels before the first interaction (Wilcoxon test: $Z = -2.33$, $p = 0.02$).

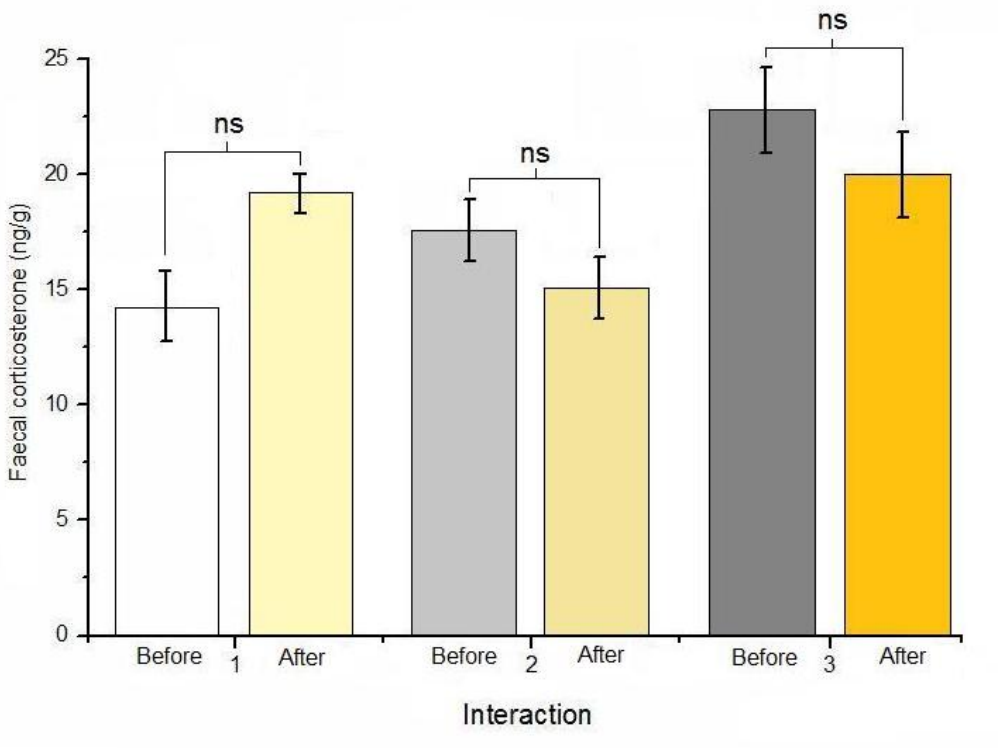


Figure 2.20 Corticosterone concentration of faecal samples collected before and after interactions.

Only corticosterone levels of samples collected before interactions differed significantly across the experiment ($\chi^2(2) = 8.00$, $p = 0.02$); levels were significantly greater before the third interaction than before the first ($z = -2.33$, $p = 0.02$). Bars represent means + SEM.

2.6 DISCUSSION

2.6.1 Context-dependent male signalling

The first objective of these experiments was to establish if the context in which females or female cues are encountered influences individual investment in male signalling. Male signalling investment varied according to female cue type. Further, cues that elicited a high rate of calling differed from those that elicited a high rate of scent marking suggesting these auditory and olfactory signals may have different communicative functions. Ultrasonic calls were primarily emitted in interactions, suggesting an important role in direct courtship and prolonging close contact with a female. By contrast, the greatest number of scent marks was deposited when males were not in direct contact with female conspecifics suggesting these cues are more likely to be important in attraction rather than serving as a functional cue during sexual encounters. Scent marking and calling therefore appear to be used in different communicative contexts. Consistent with these results, scent marks are known to provide long lasting cues that persist in the environment, providing information to competitors and potential mates even when the scent owner is absent (Hurst *et al.,* 1998). By contrast, ultrasonic calls were produced primarily during interactions, consistent with the theory that these signals are important for courtship in mice (Nyby, 1983).

These results are also consistent with the multiple messages hypothesis regarding the evolution of multiple signals, which predicts that sexual signals are adapted for use in different contexts and that together they provide a more accurate reflection of male quality (Moller and Pomiankowski, 1993). As a result, more than one signal may be needed for communication in all contexts important for mate choice decisions. Consistent with this, the results of this experiment show context-dependent investment in signalling by male mice. Females may assess males based on their scent alone, but the likelihood of choosing a mate of high quality may be improved if call rate is subsequently assessed during sexual encounters. Assessing both scent marking and call rate may provide additional benefits to females by reducing the number of males that a female needs to inspect closely (Candolin, 2003). This decreases the energy and time expenditure of females and may also moderate the risk of aggressive and risky encounters with unsuitable males. For this process to function, males must produce an initial attraction signal that females could assess before meeting a male and a subsequent courtship signal that would be used once the female has chosen to inspect an individual

more closely. For example, female satin bowerbirds (*Ptilonorhynchus violaceus*) make multiple visits to male nest sites before choosing a mate, assessing several signals including bower construction, decoration and the physical displays of a male (Borgia, 1995). Females use male size and display rate to initially assess a male from a distance; subsequently, the rate at which males 'paint' the inside of a bower with saliva and chewed up plant material is inspected more closely (Robson *et al.,* 2005). The results of the experiment presented here indicate male mice may utilise a similar system, allowing females to balance the cost of assessing multiple males with ensuring they choose the most suitable mate. Initial assessment could be made using olfactory signals in the form of scent marks, followed by assessment of male call rate during interactions.

In addition to addressing signalling in different communicative contexts, correlations between individual scent mark and calling investment were investigated. Investment in these signals was only correlated during 'barrier' trials, when calling and scent marking were both high relative to in other contexts. A similar result has been observed in other studies when male signalling is recorded in response to a range of female odour cues; signalling was only correlated in individuals exposed to particular cue types (Roullet *et al.,* 2011). This suggests social context not only influences the extent to which males invest in either scent marking or calling, but that additionally, these cues modulate whether investment in two signals is correlated. It has been suggested that investment in different signals is only correlated in the most biologically relevant contexts (Roullet *et al.,* 2011), but in the experiment outlined here, the 'barrier' trial is likely to be the least representative of a natural social situation (i.e. where individuals can see, smell and hear one another but cannot interact). However, the presence of the female on the opposite side of a barrier would provide males with limited behavioural feedback from females in response to signalling investment. In addition, the presence of the female throughout presents a potential mating opportunity. As a result, individuals may be motivated to heavily increase their investment in both scent marks and calls, reaching the limits of their ability to produce both signals simultaneously.

2.6.2 Individual testosterone levels and male signalling investment

Urinary testosterone was correlated with male signalling investment, but only when males were separated from females by a barrier and invested relatively heavily in both scent marks and calls. It is possible that hormone levels of individuals only play a significant role in mediating signal investment in contexts that elicit high investment in both marking and calling, which are known to be androgen dependent (Nyby et al., 1992; Sipos and Nyby, 1998). As a result, situational and physiological factors may interact to determine individual signal investment. Alternatively, the lack of a relationship between individual urinary testosterone and signalling in any other context may be due to one of the main limitation of this experiment; only baseline levels of testosterone were measured. As a result, any other important links between this hormone and signalling investment may not have been detected. For example, signalling investment may be greatest in individuals that experience large changes in testosterone or have the highest androgen levels following an interaction. It is also possible that the method used to analyse testosterone does not accurately reflect circulating levels in individuals at the time of sample collection. Urinary testosterone was measured and this is assumed to correspond well with circulating levels. However, the interpretation of this data may be influenced by a number of important factors, including the fact that the temporal relationship between acute testosterone surges in the circulation and the subsequent release of this hormone via excretion is likely to vary between individuals. Testosterone may also fluctuate with circadian rhythm (Lucas and Eleftheriou, 1980), and in response to being exposed to a female or female odour (Coquelin and Desjardins, 1982). As a result, individual levels of this hormone may have changed dramatically over the course of the experiment, meaning the data collected may not adequately represent individual testosterone levels before each trial; rather, it only estimates the underlying levels of this hormone in animals prior to any experimental manipulations.

A second limitation of this experiment is that other specific features of male calling were not assessed. As a result, individual testosterone may have been correlated with call characteristics that were not measured. Vocal signals in mammals are generated by converting the airflow from the lungs into acoustic energy in the larynx, which is followed by filtering in the vocal tract. The size of the vocal chords is related to the range of pitch that can be produced and the rate at which vocal folds open and close in

the larynx specifies the fundamental frequency of a call or vocalisation (Fitch and Hauser, 2003). Male vocal folds are also influenced by testosterone, so other features of auditory sexual signals may provide reliable information about the hormonal status of individuals (Beckford *et al.,* 1985). It is possible that call frequency and structure are more susceptible to differences in individual hormone levels than call rate and females respond more to changes in these features as a result. As structure and frequency were not analysed, it is unclear whether male urinary testosterone was correlated with either of these features or if the behaviour of females was influenced by factors other than time spent calling.

2.6.3 Urinary protein concentration and male scent marking

When separated from females by a barrier, males typically deposited more scent marks than in any of the other trials. In this particular context, urinary protein concentration was negatively correlated with scent mark investment; males that produced the highest number of marks had the lowest levels of urinary protein prior to the start of the experiment. Urinary protein concentration is indicative of MUP expression and these proteins are an important component of scent marks, providing information to females regarding individual and genetic identity (Cheetham *et al.,* 2007). As MUPs are likely to be energetically costly to produce (Gosling *et al.,* 2000), males may need to balance protein expression with the number of scent marks they deposit. For males that express high levels of protein in their urine, only a small number of scent marks may be required to provide adequate olfactory information to potential mates. By contrast, when urinary protein per mark is low, a greater number of scent marks may be required for these olfactory signals to be detected. However, when protein concentrations were adjusted for urine dilution, the relationship was no longer significant. This suggests that males with more dilute urine produce the greatest number of scent marks, probably due to having fuller bladders, thus enabling them to do so. This was further confirmed by the significant relationship between scent marking and creatinine, a by-product of muscle metabolism traditionally used to correct for urine dilution. Male scent mark investment may be mediated simply by the volume of urine in the bladder of an individual at the time when scent marks are deposited, rather than by

a requirement to balance any costs associated with simultaneously producing high quantities of protein and scent marks.

2.6.4 Male signalling investment and behaviour of males and females

Females prefer to associate with males that scent mark or call at a high rate (Pomerantz *et al.,* 1983; Roberts and Gosling, 2003), and male ultrasonic calls are thought to be closely linked to sexual arousal (Nyby, 1983). It was hypothesised that male signalling investment would be positively correlated with male and female behaviour during interactions. When animals were separated by a barrier, the amount of time males and females spent pulling and chewing on the barrier was positively correlated with male calling investment. These behaviours were recorded as a measure of individual motivation to directly contact the animal on the other side of the barrier. The results suggest calls encouraged females to attempt to make contact with the male and that calls indicated the level of motivation of males to access the female. This is consistent with the theory that male ultrasonic calls serve a courtship function in mice, correlate with male sexual arousal, and stimulate the interest of a female (Nyby, 1983; Hammerschmidt *et al.,* 2009).

Male and female behaviour was also measured during direct interactions. During interactions, the affiliative behaviour of males was positively correlated with calling investment and negatively correlated with scent marking; males that displayed more affiliative behaviour towards the female produced a high number of calls but few scent marks. This is consistent with the notion that calling is important during courtship. Interestingly, males displaying a high level of affiliative behaviour towards females typically mounted less and exhibited a longer latency to the first mounting attempt. Males that produced few calls attempted to mount much sooner during interactions and made more attempts overall. Differences in the mating strategies of individual males may have influenced the differences in behaviour observed. For example, when females are provided with free access to a range of potential mates, subordinate males with more limited access typically mount and ejaculate as soon as possible. By contrast, dominant males display a mating pattern more consistent with mate guarding and extensive courtship (Rolland *et al.,* 2003). This is consistent with the data obtained; males that produce few calls, which is typically associated with subordination, tended to

mount quickly. By contrast, males that produced the highest levels of ultrasound spent more time 'courting' the female and displayed longer mount latencies. However, males in this experiment were singly housed and thus the opportunity to form dominance hierarchies was removed. Despite the lack of competition between males it is possible that individuals in this experiment invested in distinct mating strategies. Alternatively, the length of time that subjects and females were permitted to interact may have influenced male behaviour. Males only encountered a single female for 30 minutes, potentially limiting the expression of sexual behaviour. Typically, male laboratory mice make multiple mounting attempts before successful copulation, so the short length of these trials may have prevented successful mating (McGill, 1962). For example, some inbred strains have been shown to require more than one hour of interaction before intromissions and successful copulation occur (McGill, 1962).

Male affiliative behaviour was negatively correlated with scent mark investment but positively correlated with affiliative behaviour in females. Females spent more time sniffing and investigating males that also expressed a high level of these behaviours but produced few scent marks. This was confirmed by a significant negative correlation between female affiliative behaviour and male scent marking. As scent mark investment was lowest on average during interactions this signal is likely to be of low importance during sexual encounters, potentially due to increased investment in calling as the predominant signal. However, the relationship between scent marking and sexual behaviour suggests females may still be able to gain important information from scent marks during interactions. As a result, when the number of scent marks deposited is low, females may seek alternative olfactory information, such as in ESPs excreted in tears and secretions from other glands (Kimoto *et al.,* 2005; 2007). For example, ESP1 produced in the tears of males is known to influence female receptivity during sexual encounters (Haga *et al.,* 2010). This would require close investigation of the male and increased sniffing of the facial area and body, potentially explaining the result observed. In addition, high scent mark rate indicates dominance and high competitive ability or motivation to defend a scent marked territory (Desjardin *et al.,* 1973; Hurst and Beynon, 2004). This may include extreme aggression and offensive behaviour directed at intruders and unfamiliar individuals, including females. As a result, females may be more cautious in the presence of males that scent mark at a high rate, avoiding prolonged direct contact to minimise the risks of injury as a result of aggressive male

behaviour. However, this data only represents a correlation between female behaviour and male signalling so does not address causality. Whether males deposit a high number of scent marks in response to low investigative behaviour from the female, or females investigate a male further if minimal olfactory information is present (i.e. due to low scent marking) is unclear.

2.6.5 Consistency in signalling investment and sexual behaviour

The second experiment was conducted to further address questions regarding the stability of individual signalling investment and to investigate the potential relationships between hormones and sexual signals. It was hypothesised that male signalling investment is a stable and consistent trait within individuals (Whitney *et al.,* 1973; Roullet *et al.,* 2011). As predicted, individual male investment in calling and scent marking was consistent relative to other males, regardless of the female that males were paired with or the behaviour of those females. This suggests signalling investment is relatively stable and female behaviour has little influence on the relative investment of males. This is in contrast to males of many species that may adjust signalling investment as a result of female behaviour. For example, male whitethroats (*Sylvia communis*) court females by diving whilst producing a specific complex song. Females produce sharp calls in response and sometimes jump towards the male and these calls and jumps influence male courtship behaviour (Balsby and Dabelsteen, 2002). This type of male response is more common in species that 'duet' during courtship and form pairs for prolonged periods, particularly where female produce signals that are comparable to those produced by males in terms of specificity and variability. Males that are attentive to these cues could reduce their costs of producing signals by limiting time and effort and reducing the risk of aggressive rejections. This would be of particular importance for species that produce signals that are costly in terms of energy or are highly conspicuous, thus leading to increased predation risk and male competition. Alternatively, when there is little cost to producing a signal, males may invest heavily regardless of female behaviour. In the experiment outlined here, perceived costs of male competition are low due to the absence of other males, a factor that may influence male signalling investment in mice; when exposed to females in the presence of urine from other males, ultrasound production by the focal male is decreased (D'Amato, 1991). As

a result, males in this experiment may not have altered their calling investment but simply signalled at a high rate when a female was encountered. Additionally, in the laboratory males are released from the potential energy constraints of investing heavily in signalling as physical activity is confined to a small area and food and water is available *ad libitum*.

Despite the signal investment of individual males remaining consistent relative to other individuals, calling investment for males as a group increased over the course of the experiment. As the calling behaviour of males remained consistent relative to other individuals in the group, this suggests that all males increased their calling to some extent as a result of their prior experience, and that the increase was relatively similar for all males. As a result, simply encountering a female, regardless of the behavioural response of that female or the motivation of the male, may influence male calling investment in subsequent encounters. Behavioural data was collected from three interactions for each male and female; using the same statistical methods as in experiment one, the primary patterns of behaviour were obtained via principal components analysis. The first component obtained for male behaviour contrasted affiliative behaviours with the number of mounting attempts. Scores for this component decreased significantly across the three interactions, suggesting that male behaviour became less affiliative towards females and the number of mounting attempts increased (mean of 1 attempt during interaction 1; mean of 5 attempts during interaction 3). This is consistent with previous reports of sexual behaviour during multiple interactions in another laboratory strain of mouse; experienced males decreased their exploration of females prior to mounting attempts and exhibited lower mount latencies (Swaney *et al.,* 2012). In addition, studies in other rodent species revealed that behavioural components of copulation occur with shorter latencies and higher frequency in sexually experienced males (Dewsbury, 1969; Coopersmith *et al.,* 1986). It is therefore possible that prior experience allows males to respond to females more quickly and become more sexually 'competent' (Swaney *et al.,* 2012). In addition, olfactory investigation of the female was reduced over the course of the experiment, suggesting less olfactory information is required to initiate sexual behaviour in experienced males. In support of this, detection and investigation of female odours is known to initiate courtship, and disruption of the olfactory system in males disrupts

sexual behaviour (Mandiyan *et al.,* 2005), although these effects are less prominent in sexually experienced males (Pfeiffer and Johnston, 1994).

The first component obtained from the PCA on female behaviour contrasted affiliative behaviour towards males (sniffing, grooming, following) with the number of times females fled from mounting attempts. Similarly to male behaviour, mean scores for this component declined over the course of the experiment, suggesting females displayed less affiliative behaviour towards males in the latter interactions. This may simply have been a negative response to the increased number of mounting attempts made by males due to the fact that pacing is an important component of sexual behaviour in female rodents. Pacing refers to the intermittent approaches and withdrawals from a male that allow females to control the pace of coital stimulation and interaction (Erskine, 1989). Studies of this component of sexual behaviour in laboratory rats show that during prolonged periods of testing in small arenas, lordosis and female receptivity is inhibited after intense mounting activity by males; further, rejection by females is likely to increase (Hardy and DeBold, 1972). In contrast, females react more positively to sexual encounters when they are able to control the pace of interactions in larger, semi-natural environments (Paredes and Vazquez, 1999). As the test arena in this experiment was relatively small and male mounting behaviour intensified in later interactions, it may have been increasingly difficult for females to pace the rate of the interaction due to there being little opportunity to retreat. This could have resulted in lower receptivity and increased rejection of male advances, explaining the decline in affiliative behaviour in later interactions. Another factor that may have influenced female receptivity is the prior experience of females when juvenile. Female rodents reared by mothers that display a low level of licking and grooming towards their pups are likely to be more sexually receptive to males in adulthood (Cameron *et al*., 2008). However, despite several females in this experiment being derived from the same litters, the behaviour of females was not consistent among sisters (e.g. one female rejected 11 of 11 mounting attempts during the third interaction; the sister of this female only rejected 2 of 12 mounting attempts during the third interaction), suggesting this factor is unlikely to have strongly influenced the patterns of behaviour observed.

2.6.6 Male calling as a reliable indicator of fertility

Of the sexual signals male mice produce, scent marks provide information to females regarding male quality but little is known about the functions of male ultrasonic vocalisations in mate choice. This signal has been shown to be under the control of androgens and is highly variable between individuals (Nunez *et al.,* 1978; Holy and Guo, 2005). Testosterone is also important for fertility and the development of normal reproductive physiology in males, so it is possible that a testosterone-mediated signal such as ultrasonic calls may reliably signal sperm count. The results indicated total sperm count was positively correlated with male calling investment; males that called at a high rate typically had a higher sperm count. This provides some of the first evidence for a link between this specific aspect of male quality and calling investment in mice. However, within the scope of this experiment the influence of individual sperm number on fertility and reproductive success could not be addressed. Although females typically prefer to associate with males that call over males that fail to vocalise (Pomerantz *et al.,* 1983), it is unclear whether females prefer to mate with males that call at the highest rates. Future work exploring the reproductive success of males that differ in call rate may provide useful information on the reproductive benefits, such as an increase in offspring number or fitness that females could gain as a result of choosing a male based on individual calling investment.

Surprisingly, urinary testosterone was not correlated with sperm count or any other measures of reproductive physiology. A number of factors may have influenced these results, including the times of urine collection and the analysis of urinary testosterone rather than plasma levels. To reduce the potential influence of sexual interactions on sperm production, which takes approximately 30 days (Oakberg, 1956) all interactions and urine collection took place within a 3 week period. As a result, sperm counted at the end of the experiment may have only been minimally influenced by the hormones of individuals at all the specific times during the experiment when urine was collected. In addition, over time, excretion of testosterone typically shows two to three elimination phases resulting in approximately 90% of testosterone being excreted within 24 hours (Billiti *et al*., 1998). As a result, the time between interactions and subsequent urine collection (48 hours) may not have been sufficient or optimal for detection of changes in testosterone levels as a result of interactions.

2.6.7 Male investment in scent signals and accessory gland size

The weight of male seminal vesicles was negatively correlated with the percentage of darcin relative to other MUPs. The seminal vesicles are accessory glands important in the production of seminal fluid proteins such as those involved in copulatory plug formation (Bradshaw and Wolfe, 1977). The production of these proteins, as with MUPs, may represent a high energetic cost to males (Gosling *et al.,* 2000). As a result, urinary protein and the size of accessory glands may be correlated due to a potential need to balance the costs of producing scent mark components and accessory gland proteins. The results suggest males producing high levels of seminal vesicle fluid expressed a lower percentage of darcin (relative to other MUPs) compared to other males. Although seminal vesicle and urinary proteins such as darcin are synthesised in different regions of the body (the seminal vesicle and liver respectively), their production is likely to impose energetic costs. As a result, high investment in both darcin and seminal vesicle proteins may not be possible and social context is likely to influence how males balance investment in proteins important for pre- versus post-copulatory competition. As scent marks and darcin in particular are important for attraction and learning of male scent locations (Roberts *et al.,* 2012), and thus pre-copulatory competition, the importance of darcin during sexual interactions may be minimal. If production of this pheromone is particularly costly, it is possible that males could reduce their proportional investment in darcin allowing an increased investment in other proteins such as those in the seminal vesicles, which are important in post-copulatory competition. For example, male *Drosophila* adjust the composition of seminal fluid according to perceived sperm competition risk (Wigby *et al.,* 2009). In this example, altered investment was not influenced by the presence of females, but male mice may invest differentially in the production of important components according to the perceived social environment. For example, a significant negative correlation between sperm count and scent mark rate has been observed in male mice under competitive conditions (Ramm and Stockley, 2009), possibly due to a trade-off between investment in olfactory signals important for territory defence and investment in sperm production. However, it is unclear whether a trade-off in production of these proteins occurs, so further work exploring this is needed.

In addition, no relationship between individual preputial gland size and scent marking or urinary protein concentration was found. Volatile secretions from the

preputial gland are added to urine during scent marking and the pheromonal components of these secretions are attractive to females (Bronson and Caroom, 1971; Novotny *et al.,* 1984). Previous studies have shown preputial gland size is unaffected by the presence of females but does change in response to social status; dominant males typically have heavier preputial glands than their subordinate counterparts (Bronson and Marsden, 1973). All males in this study were housed singly, so the necessary cues, stimulation or opportunity to form dominance hierarchies were absent. As a result of minimal differences between males in social status, variation in preputial gland size was low, confirmed by a mean weight of 0.18g and a standard error of only 0.01g.

2.6.8 Male signalling and the stress hormone, corticosterone

The links between the adrenal hormone, corticosterone, and male signalling investment or sexual behaviour were investigated. Faecal samples were collected from subject males before and after interactions with females to determine if baseline levels or changes in this adrenal hormone are linked to male signalling and courtship. In some rodents, stress is linked with a reduction in sexual activity and motivation (Blanchard and Blanchard, 1991). The typical preference of females for male odour is also removed when corticosterone is administered to male mice (Kavaliers and Ossenkopp, 2001). It was therefore hypothesised that males with an elevated stress response and higher levels of corticosterone would express low sexual motivation, a lower rate of signalling and that females would show less interest in these males. The results show that over the course of the experiment, baseline levels of corticosterone differed significantly, increasing as the experiment progressed. This may be due to an elevated stress response as a result of previous unsuccessful sexual encounters or to stimuli including caging conditions. However, it is unlikely that external stimuli such as caging and cleaning routines influenced this, as these were kept consistent throughout the experiment. Surprisingly, no relationships between individual corticosterone levels and sexual behaviour or signalling investment were found. Despite the lack of a correlation between male corticosterone and either male or female behaviour, it is possible that the higher individual corticosterone levels in the later interactions exerted a subtle effect on female behaviour. This may account for the reduction in affiliative behaviour displayed by females over the course of the experiment.

2.6.9 Conclusions

The overall aim of this study was to investigate why male mice invest in multiple sexual signals and what factors influence investment in different social contexts. In the first experiment, investment in scent marking and calling was recorded in a range of contexts to address why males produce two different sexual signals and how investment according to social context may be linked to individual testosterone levels or investment in specific scent signal components. The second experiment was conducted to extend the findings of interaction trials in the first experiment and to establish whether male investment in signalling is a stable trait or influenced by the behaviour of different individual females.

The results of these experiments show that male signalling investment is influenced by a wide variety of factors, including social context, individual hormone levels and the need to balance production of signal components that impose high energetic costs. Whilst many of these factors were correlated with the time males spent calling or the number of scent marks produced, the effects of many of these factors were linked. For example, both calling and scent marking were relatively high during 'barrier' trials in the first experiment; this was the only context in which baseline testosterone and urinary protein were linked to signal investment. Social context is also likely to have mediated the relationship observed between seminal vesicle size and proportional investment in darcin. High investment in both darcin and seminal vesicle proteins may not be possible due to energetic costs, creating a need to balance investment in proteins important for pre- versus post-copulatory competition. As seminal vesicle proteins are more important for post-copulatory competition, preferential investment in these proteins may occur as a result of sexual encounters. All of these results highlight the importance of taking into account the context in which sexual signalling is quantified when assessing the effects of a variety of other factors on investment.

In terms of the relationship between sexual behaviour and male signalling, the results show that signal investment of individual males during sexual encounters may be linked to the behaviour of both females and males themselves. However, differences between males in behaviour and signalling appeared to be related to mating strategy; males that mounted the most typically called the least, but displayed the least affiliative

behaviour towards females. Male signalling was also stable relative to other individuals across multiple interactions, suggesting investment in signals during sexual interactions is unlikely to be influenced by the response or receptivity of females. The level of affiliative behaviour of both males and females declined over the course of the second experiment, highlighting the importance of prior experience for sexual behaviour in both sexes. Finally, a positive correlation between calling and sperm count was found. As calling is relatively stable, it may provide females with a consistent, reliable indicator of male quality and thus be important for mate choice.

Overall, the results of these two experiments indicate that many factors influence male signal investment, but that no single factor is likely to mediate investment alone. Hormone levels, social context and the costs associated with expression of specific signal components may all interact to mediate investment in signals important for attraction of females and courtship. Despite the potential influence of several factors across multiple social contexts, within the context of sexual interactions, male signalling is relatively stable, potentially providing females with a consistent, reliable indicator of male quality. By contrast, sexual behaviour is highly variable and may be influenced by a combination of factors including prior experience.

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CHAPTER 3: Female learned spatial preferences for locations of competing male scents

3.1 INTRODUCTION

Learning is often compared to natural selection in that it 'improves' behaviour in terms of its effectiveness for meeting challenges associated with reproduction and survival. Learning can occur in a range of contexts and has the ability to influence behaviours important for habitat use, foraging, mate choice and social interaction. Social learning, which typically involves learning behaviours from conspecifics by observation, can include changes in an individual's behaviour following encounters with conspecifics or conspecific cues. In the context of mate choice, this may include gaining information that alters the response of females to specific males, improving their ability to select the most suitable mate available. Females can gain material, proximate and genetic benefits from choosing among males, but the net benefits of mate choice may be reduced by costs associated with searching for mates for extended periods of time. To maximize the benefits of mate choice, females should therefore make assessments of male quality as accurately as possible and use a mate-searching tactic that limits search costs.

Many species, including mice, utilise scent for communication with conspecifics. Unlike auditory or visual signals, reliable signals of quality and identity provided by scents are long lasting and can persist in the environment even when the scent owner is absent. Males may invest heavily in competitive marking within their territories and the distribution of male scent marks can provide an honest signal of an individual's ability to defend his territory. Females can assess male scents in terms of quality and distribution, allowing them to gain information important for subsequent mate choice. In a natural context, females would assess multiple male scents across a potentially wide search area, with scents often being present in close proximity to one another. When searching for mates, assessing the olfactory signals provided by male scent whilst simultaneously learning about the spatial attributes of scent locations would be highly advantageous, allowing females to be selective and return to the sites of preferred male scents when ready to mate.

3.1.1 Learning

Lasting changes in behaviour resulting from prior experience can be characterised as the products of learning and memory. Learning is often compared to

natural selection in that it 'improves' behaviours important for reproduction and survival. Despite this parallel, natural selection typically acts between generations and changes behaviour over long periods of time, learning is able to shape behaviour within a generation or within a single individual (Pearce, 2008). Learning can occur in many different situations and has the ability to influence a range of behaviours including those important for habitat use, foraging, mate choice and social interaction.

Learning can be grouped broadly into one of two categories: associative learning, which includes classical and operant conditioning, and non-associative learning. Associative learning typically involves the pairing of a specific stimulus, event or action and a resulting consequence. If the consequence reliably occurs every time the stimulus is encountered, an association between the stimulus and the consequence is formed and behaviour may be altered accordingly. This type of associative learning can be described as classical or operant. Classical conditioning theory is based on work by Pavlov in the early twentieth century (Pavlov, 1927), in which a stimulus that does not initially cause a specific response can be conditioned to do so by associating it with another stimulus that does initiate a response. In Pavlov's classic experiment, the salivation of dogs in response to the presence of meat was conditioned to the sound of a ringing bell (Pavlov, 1927). Initially, the presence of meat stimulated an instinctively unconditioned salivation response. After pairing the presentation of meat with a ringing bell, dogs would salivate as a conditioned response to the ringing bell alone. Pavlov also found that the response to the bell could be generalised, such that dogs conditioned to salivate in response to a specific tone would still salivate in response to other similar, but distinct tones. However, with extensive training, the dogs could learn to discriminate between similar stimuli and respond appropriately. Extinction of a conditioned response may also occur; many factors influence extinction and resistance to extinction is often used as an index of the strength of learning (reviewed by Baeyens *et al.,* 1995).

Operant conditioning involves the outcome or consequence being associated with the behaviour itself, rather than an inanimate stimulus (Skinner, 1937; reviewed by Staddon and Cerutti, 2003). The animal typically becomes conditioned through chance reinforcement of a previously non-reinforced behaviour. For example, an animal searching for food is likely to perform a wide range of behaviours. If performing one of these behaviours leads to acquisition of food, the animal will learn to perform that

behaviour when it requires food. In a classic experiment conducted by Skinner (1938), rats were trained to press a lever to acquire food or water. In this case, at the beginning of the experiment the rats would be placed in a chamber containing the lever and the lever may be knocked or pushed during normal investigatory behaviour; food or water would then be provided to the animal as a result. Over time, the animal would associate pressing the lever with acquisition of an important resource and subsequently push the lever when food or water was required. The food reward therefore acts as a reinforcing stimulus, which Skinner (1937) defined as "any stimulus which, when made contingent upon a class of behaviours, increases the future probability of the occurrence of a member of that class of behaviours".

Non-associative learning may involve the acquisition and retention of information that was previously unknown, allowing an animal to improve its response to a specific situation in the future. Habituation is considered an important form of non-associative learning, and has been defined as "learning what not to do" (Razran, 1971). Animals must constantly process vast amounts of information from the environment and conspecifics. Responding to every conspecific signal or environmental change would be costly in terms of energy and time, so it would be beneficial for animals to only respond to the most important stimuli. Habituation learning provides individuals with a method of filtering information and reducing or eliminating a response to a signal that may be important, but in the current situation is not (reviewed by Rankin *et al.,* 2009). The primary index of habituation is therefore a decrement in response to repeated stimulation or encountering the same stimulus repeatedly (Thomson and Spencer, 1966).

Social learning is a specific form of learning, and can include changes in an individual's behaviour being influenced by observation or interaction with conspecifics or conspecific cues (reviewed by Heyes, 1994). This may include forms of both associative and non-associative learning. For example, female assessment of individual males and acquisition of information regarding the quality of a potential mate from sexual signals is a form of learning important in mate choice. This information can alter the behaviour and response of females to specific individuals, improving their ability to select the most suitable mate available. Further, forming an association between important, rewarding social cues and their locations within the environment may be important for subsequently locating these suitable individuals. Mating preferences can

also be learned through prior experience with conspecifics themselves (reviewed by Verzijden *et al.,* 2012). For example, several species develop learned preferences for individuals expressing a specific phenotype with which they have had a previous successful mating experience (e.g. Magurran and Ramnarine, 2004; del Barco-Trillo *et al.,* 2010; Takahashi and Watanabe, 2009). In some cases, experience with individuals expressing many different variations of a sexual signal or trait is required for a preference to develop; female wolf spiders (*Schizocosa* spp.) from polymorphic populations only prefer ornamented males after experience with both ornamented and unornamented individuals (Hebets and Vink, 2007).

Beyond mate assessment and learning of preferences for specific variations of a sexual signal, learning where potential mates are located is an essential part of mate choice. Females can gain material, proximate and genetic benefits from choosing among males (Andersson, 1994), but the net benefits of mate choice may be reduced by costs associated with searching for mates. Mate searching behaviour may therefore precede mate choice and determine the number of high quality potential mates encountered. As a result, this behaviour can have a significant impact on reproductive fitness. To maximize the benefits of mate choice, females need to choose among males and use a mate-searching tactic that limits search costs (reviewed by Janetos, 1980). The importance of a reliable and rapid method for mate searching is further intensified if individuals are only able to breed during certain seasons or at certain times (e.g. due to fluctuations in hormones during the oestrus cycle). The relatively high costs associated with extensive searching should encourage prioritisation of efforts to locate individuals of the highest quality and avoid searching for individuals that would be unsuitable mates. Discrimination between potential mates based on sexual signals that are encountered before a male himself (e.g. scent marks) is therefore an important component of reproductive fitness in species where females search for suitable mates (Real, 1990; Jennions and Petrie, 1997).

3.1.2 Female learning and assessment of competing males

Sexual selection is underpinned by the notion that competitively superior males are likely to confer the greatest indirect or direct fitness benefits to the females that choose them as mates (reviewed by Andersson and Simmons, 2006). Females may be

able to make more informed choices and gain increased fitness benefits by choosing males based on competition or competitive signalling, particularly in cases where males use the same signals in male-male competition and attraction of potential mates (reviewed by Hunt *et al.,* 2009).

Most studies investigating sexually selected traits and the factors that influence male signalling focus on either intra- or inter-sexual selection. However, in many species, male signals used for competition with other males also serve as important signals for female attraction and mate assessment (Berglund *et al.,* 1996). As a result, competitive signalling between males may alter individual investment and thus influence female choice. Additionally, if signalling under competitive conditions is costly, signal honesty may be increased, thus facilitating more accurate female assessment of males, particularly in species where dominant males of high quality are likely to suffer fewer costs when increasing signal rate or intensity as a result of competition. The importance of competitive signalling for female choice has been documented in several species when changes in signalling intensity as the result of male-male competition influence female choice (e.g. Morris *et al.,* 1995; Galeotti *et al.,* 1997). For example, male competition influences the intensity of red coloration of male three-spined sticklebacks (*Gasterosteus aculeatus*). This in turn facilitates female choice and allows individuals to more reliably select the most dominant males (Candolin, 1999). Similarly, the black facial mask of common yellowthroats (*Geothlypis trichas*) serves a dual function in male-male competition and female mate choice (Tarof *et al.,* 2005).

Adult male mice invest heavily in competitive territorial scent marking, a behaviour that plays a role in conspecific communication in many species (Johnson, 1973). Production of scent marks is often physiologically costly and high investment may lead to reduced growth rate and small body size (Gosling *et al*., 2000). This cost is thought to help maintain honesty in signalling, as only high-quality individuals can afford to make high investments in these secondary sexual signals (Zahavi, 1977). Females can gain information from male scents and learn about the quality of a male with regards to his health and social status (Gosling and Roberts, 2001). For example, male status can be assessed through several of the androgen-dependent volatile components that are added to male urine when scent marks are deposited (Bronson and Caroom, 1971; Jemiolo *et al.,* 1985). Suppression of some of these components such as the farnesenes produced in the preputial gland occurs in subordinate individuals,

making their scents unattractive, thus influencing the response of females to individual marks (Jones and Nowell, 1974). In addition, infection and increased parasite load may render male scents unattractive (Penn *et al.,* 1998; Ehman and Scott, 2001), allowing females to avoid infected males.

As introduced previously in section 1.3.2, the location and distribution of male scent marks is also important for female assessment, as this accurately signals details of the social status and competitive ability of an individual. Males increase their rate of scent marking and the expression of specific scent mark components such as MUPs under competitive conditions (Garratt *et al.,* 2011), potentially providing females with a more intense signal of male quality and facilitating assessment of individual males. Females are typically more attracted to males that scent mark at a high rate (Roberts and Gosling, 2003), and strongly prefer the odours of dominant males that own scent-marked territories (Mossman and Drickamer, 1996). Dominant males typically deposit a combination of large and small urine streaks at numerous sites around their territory (Desjardins *et al.,* 1973), often utilising specialised hairs on the prepuce to increase the area covered by each streak (Maruniak *et al.,* 1975). Males advertise territory ownership by maintaining exclusive scent-mark coverage of their home ranges (Rich and Hurst, 1999), and advertise their social dominance via countermarking (Rich and Hurst, 1998), which involves a rapid increase in the rate of marking and depositing of scent next to the marks of intruders or subordinates (Humphries *et al.,* 1999). The scents of other males may represent a challenge for dominance and can create ambiguity for females when establishing the identity of the territory owner (Gosling, 1982). By countermarking all other male scents, individuals are able to reinforce their dominance and ensure their scent remains the most recently deposited. This is of particular importance as females prefer the owners of exclusively marked territories over those whose territories contain competing marks from neighbouring males (Rich and Hurst, 1998). Signalling dominance through scent is therefore an important aspect of scent mark investment for males, and scent mark rate and distribution provide valuable information to females regarding male competitive ability and territory ownership.

For females to utilise information learned about males in subsequent mate choice decisions, recognition of individual male scent owners must occur. The information signalled by male scent must therefore include fixed components that remain stable over time and are not influenced by changes in health or social status. The

composition of scent marks signals genetic relatedness or identity in several species (e.g. Lawson *et al.,* 2000; Charpentier *et al.,* 2008) and in mice, the major urinary proteins (MUPs) present in scent marks provides an individual and genetic olfactory signature (Hurst *et al.,* 2001; Cheetham *et al.*, 2007). Mice typically express between 8 and 12 different MUPs within their urine, each in different ratios depending upon the individual. This provides an opportunity for extremely high diversity in the MUP profiles of individuals (Beynon *et al.,* 2002), allowing not just discrimination and assessment, but also learned recognition of specific individuals (reviewed by Hurst, 2009). MUPs are not only genetically stable, but are highly resistant to degradation and can persist for many weeks once deposited in the environment. This provides a persistent and reliable olfactory signature that allows females to not only recognise specific individuals, but to assess familiarity over protracted periods of time. This may be important as females may regularly come into contact with the odour signals of some males whilst encountering others infrequently or not at all. Females find the owner of a familiar scent more attractive than the scent of a completely novel individual (Cheetham, 2006), and there are a range of potential benefits to this preference. For example, assessment of long term male quality in terms of health status and competitive ability may be more accurate if a male or his scent has been encountered several times (Cheetham *et al.,* 2008). This would provide information regarding the ability of a male to maintain his health and dominance status over a protracted period of time, representing a more honest view of overall quality.

MUPs mediate inherent attraction to male scent (Martinez-Ricos *et al.,* 2008), and associative learning of the volatile scent signature of individuals is possible following direct contact with a scent and detection of the involatile component (Ramm *et al.,* 2008). After this initial contact, females are able to identify individual males on the basis of the associated volatile components alone, a process mediated by the male-specific MUP, darcin (Roberts *et al.,* 2010). As volatile signals can be detected at a distance, female may be able to minimise energy and time expenditure required for full investigation of every male scent they encounter (**Figure 3.1**). This provides a mechanism by which attraction to male scent marks can be inherent as well as selective, thus providing a flexible response to the specific scents of individuals through associative learning.

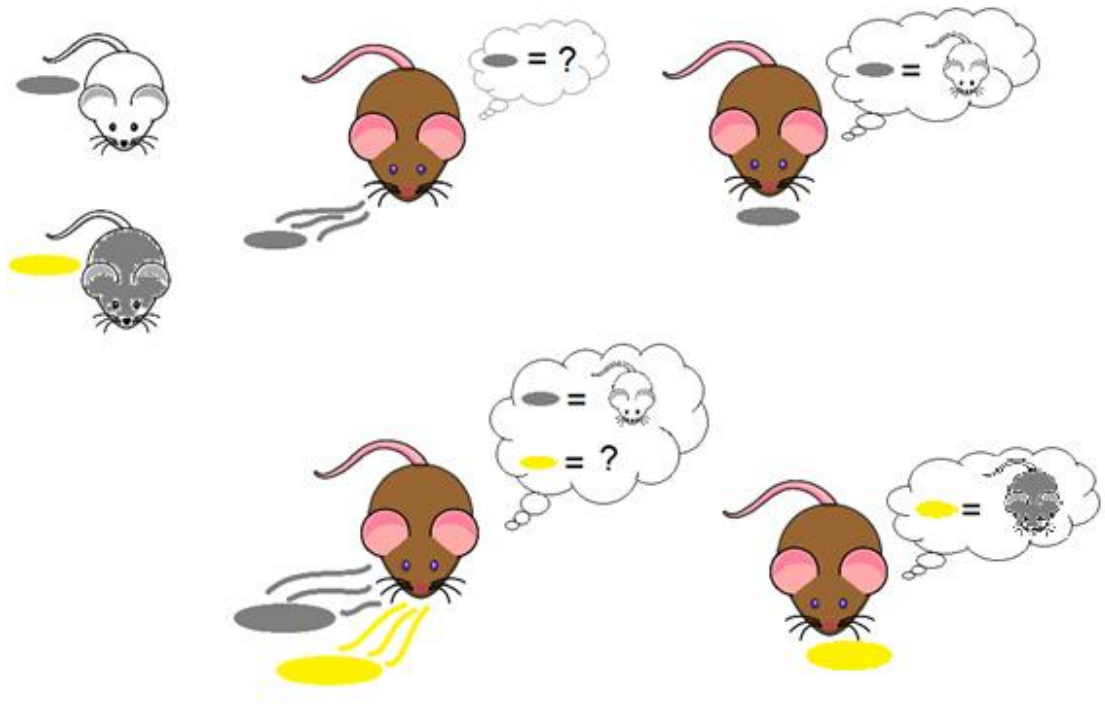

Figure 3.1 Associative learning of volatile scent signatures through direct contact.

Volatile scent components are more readily detected at a distance and would not require close investigation if the information signalled was familiar. If unfamiliar, the receiver could approach and contact the mark to obtain further information regarding the scent owner. (Adapted from Hurst and Beynon, 2004).

For female assessment and learning of individual male scent signatures to be useful in subsequent mate choice decisions, acquiring a memory of the location where a specific scent was encountered would be important, as males typically scent mark within their territorial ranges. In a natural context, females would encounter and assess multiple male scents across a potentially wide search area, with scents often being present in close proximity to one another. The ability to form an association between an attractive, high quality scent and its location would be highly advantageous, allowing females to be selective and return to the sites of preferred scents when ready to mate; accurate spatial learning in combination with olfactory assessment of males would allow females to reduce costs associated with extensive mate searching.

3.1.3 Spatial learning

Spatial learning and the acquisition of spatial memory is the part of functional memory responsible for recording information about the spatial environment with regards to other objects or cues. In humans and a range of mammals spatial memories are thought to be summarised in the brain as a cognitive map (O'Keefe and Nadal, 1978), or a mental representation that allows acquisition, storage and retrieval of information about the relative locations of objects or cues. A cognitive map is "a record in the central nervous system of macroscopic geometric relations among surfaces in the environment used to plan movements through the environment" (Gallistel, 1990). One major property of this map is that it encodes information about the spatial relationship between objects in the environment. If animals possess a map of this type, then the user should still be able to navigate to a goal location even if it its current location is unfamiliar. An influential experiment by Morris (1981) was conducted to demonstrate that rats use cognitive maps. In this experiment, rats were trained to swim to a submerged platform that could not be seen, within a vat of milky water. The apparatus was located in a room with a number of distinctive features that the subjects could potentially use as landmarks. After being released from the same location in the vat multiple times, rats quickly learned to swim directly to the platform, which always remained in the same position (Morris, 1981). Even when rats were subsequently released from a different location in the vat, they were able to navigate to the

submerged platform without too much difficulty, albeit more slowly than when released for the original training location (Morris, 1981).

Specific areas of the brain are associated with spatial acquisition and recall, including the hippocampus (Eichenbaum *et al.,* 1999), which contains specific neurons called 'place cells' that are particularly important for spatial memory (O'Keefe and Dostrovsky, 1971). The stimulation and excitation of these cells depends upon an animal's location within the environment and the 'place field' is the region of the environment where a given place cell will fire (reviewed by Muller *et al.,* 1991). The evolution of brain structures important for spatial learning may be linked to the complex home ranges and territories used by mammals and birds (reviewed by Benhamou and Poucet, 1996). In addition to the demands of maintaining home ranges for predator avoidance and foraging, there are considerable evolutionary pressures to form accurate spatial memories when searching for suitable mates or to remember the location of safe sites more generally. There may also be further pressures on species that invest in parental care, as offspring are not always able to follow parents when foraging; an accurate spatial memory of a nest site is therefore essential.

Spatial information is typically processed egocentrally or allocentrically. Egocentric processing enables memorisation of a target goal or location in relation to its position relative to the individual. This involves continual updating of the target location relative to movement within the environment. The ability of animals to keep track of a target location during search behaviour has been demonstrated in a number of species (Mittelstaedt and Mittelstaedt, 1982; Etienne *et al.,* 1991; Etienne and Jeffery, 2004), and in order to return to an important location in the future, the configuration of specific landmarks from the current location of an individual must be compared to the configuration previously memorised, a process commonly known as path integration (reviewed by Etienne and Jeffrey, 2004). Path integration is a method of navigating where no reference is made to external landmarks. Instead, a record is kept of one's position in respect to some reference point by taking account of the distance that has been travelled and the changes in direction that have been made. This type of processing has been successfully demonstrated in a number of species (Kavaliers and Galea, 1994; Kimble and Whishaw., 1994), and the ability of rodents to remember the locations of several food-caches has been shown to involve this type of processing (Jacobs, 1992). It may also be particularly useful in locating grouped or nearby targets by

performing search loops around the originally memorised location. For example, female sheep (*Ovis aries*) use a search loop strategy when searching for grouped feeding bowls (Dumont and Petit, 1998). Similarly, desert ants (*Cataglyphis fortis*) perform search loops when searching for burrow entrances (Muller and Wehner, 1994). This type of behaviour could be important when foraging in a patchy environment or for mate choice when searching for an individual around a location where a conspecific signal was previously encountered. Although egocentric processing is important for spatial memories, it is unlikely to mediate memory formation alone. Rather, a combination of egocentric and allocentric processing would enable individuals to form the most accurate spatial memories (Burgess, 2006), with allocentric processing defining the target location with reference to the configuration of other landmarks within the environment.

In the natural environment, animals engage in a wide variety of behaviours that rely on spatial navigation, learning and memory. These include, but are not limited to, homing, migration, foraging, territory defence and locating conspecifics and potential mates. These behaviours are essential for reproduction and survival and in many cases rely on the function of a region of the brain known to be important for spatial navigation and learning, the hippocampus. Many studies of hippocampal function and learning in a natural context attempt to understand food storage and retrieval in a range of species that participate in food caching. This type of behaviour is particularly useful for many species of birds, especially members of the Corvidae (e.g. crows, jays) and Paridae (e.g. chickadees) families that tend to store food in multiple locations on a seasonal basis, creating caches in the autumn and retrieving items in the winter when food is more scarce. This behaviour is essential for winter survival, so accurate spatial learning of food storage sites is vital. This type of spatial learning has been demonstrated in many species (reviewed by Raby and Clayton, 2010); further, dependency on cached food is correlated with performance in tasks that require spatial memory in a laboratory setting when the capability of multiple species is compared (Kamil *et al.,* 1994; Olson *et al.,* 1995). For example, Balda and Kamil (2006) found differences in reliance on food-storing across four American corvid species accurately predicted the performance of individuals in specific tests of memory including cache retrieval accuracy and radial maze performance.

This type of spatial learning and association between food sources and their locations within the environment has been shown to require the hippocampus, an anatomical region of the brain associated with many functions including spatial learning, episodic memory and emotion (Jarrard, 1995). When the hippocampal volume of a range of avian species was quantified, the volume of this brain region relative to overall brain and body size was significantly larger in bird species that store food and create caches than in those species that do not (Krebs *et al.,* 1989). Further, hippocampal size appears to be correlated with the degree of food-storing behaviour (Lucas *et al*., 2004), and lesions to the hippocampus and disruption of its function impair cache recovery in several species (Krushinskaya, 1966; Hampton and Shettleworth, 1996). Seasonal increases in hippocampal volume have also been shown to coincide with periods of the most intensive caching (Smulders *et al.,* 1995), suggesting enhancements in this region of the brain may be important during periods when pressure on accurate retrieval of spatial memories is at the greatest level. In addition, neurogenesis, or the recruitment of neurons to the hippocampus, typically occurs at greater levels in food-storing birds and may coincide with periods preceding the peak in storing behaviour (Barnea and Nottebohm, 1994; Hoshooley and Sherry, 2007; Sherry and Hoshooley, 2010).

Spatial learning is important in a natural context for other behaviours such as navigation to nest sites and locating conspecifics. Home range navigation is well understood in birds, but spatial learning in mammals is less well studied. Territory size is often sexually dimorphic and as a result may impose different spatial learning requirements on individuals of the same species. For example, adult male meadow voles (*Microtus pennsylvanicus*) have home ranges that are significantly larger than those of females or juveniles (Gaulin and Fitzgerald, 1986). This suggests that males are required to have superior spatial learning and memory skills than females. In support of this, male meadow voles outperform female conspecifics in maze-learning tasks, a sexually dimorphic response not observed in other rodent species where males and females have similar size home ranges (Galea *et al.,* 1995; Kavaliers *et al.,* 1998). In addition, males of this species have greater hippocampal volumes than females (Jacobs *et al.,* 1990) supporting the notion that hippocampal function is essential for spatial learning in a natural context. Animals that require an enhanced ability to navigate and learn about the spatial attributes of their environment may have evolved larger hippocampal regions to cope with this challenge.

3.1.4 Combined olfactory and spatial learning

Female mice are attracted to investigate male scents, and these scents can condition a preference for their location (Martinez-Ricos *et al.,* 2007), stimulated by a specific sex pheromone in male urine (Roberts *et al.,* 2012). This spatial memory may be important for females when searching for suitable mates, as males invest heavily in scent marking and the distribution and pattern of scent reliably indicates territory ownership and competitive ability (Rich and Hurst, 1998). Following initial assessment of multiple male scents, the ability to form an association between an attractive, preferred scent and its location would be highly advantageous, allowing females to be selective and return to the sites of preferred scents when ready to mate.

Olfactory information has been shown to be important in enhancing spatial navigation, particularly in young rodents when visual acuity is not yet fully developed. For example, mouse pups can be trained to navigate back to their nest using the spatial orientation of olfactory cues (Wiedenmayer, 2000). This process is impaired if hippocampal function is disrupted, despite odour discrimination ability remaining intact, suggesting combined olfactory and hippocampal processing is required for rodents to form a spatial representation of odours in the environment. In adults, once visual information can be processed more accurately, the presence of olfactory cues can enhance spatial learning even if the cue is subsequently removed (Lavenex and Schenk, 1996). Further, disruption of olfactory bulb function impairs navigation in spatial memory tasks, suggesting olfactory processing provides essential support for spatial navigation (reviewed by Jacobs, 2012). Forming an association between olfactory cues and their spatial location would be highly advantageous beyond improvements in overall navigation, particularly in the context of mate choice when females must locate potential mates.

3.1.5 Measuring olfactory and spatial learning

When searching for mates, females may discriminate between and assess the olfactory signals present in male scent whilst simultaneously learning about the spatial attributes of these scent locations. One of the most straightforward techniques for assessing discriminability between odours and olfactory learning is the habituation-

dishabituation test, which has been used to establish the ability of rodents to distinguish between and learn a wide variety of social odours (reviewed by Beauchamp and Yamazaki, 2003). The habituation–dishabituation assay consists of presenting subjects with consecutive, identical odours followed by a second, different odour. Subjects typically sniff the first odour with high frequency, but as they become familiar with the odour, their investigations decline, resulting in habituation. If the sniffing behaviour of subjects increases upon the presentation of a second, different odour, this "dishabituation" response indicates subjects are able to detect the change in odour. The habituation behaviour forming an essential component of these tests is widely regarded as being among the most simple and pervasive forms of learning (Schwartz, 1984); as olfactory cues are important for communication in mice, the investigation of conspecific odours that have not previously been encountered is likely to be useful in many different contexts. In addition, these tests provide a measure of olfactory learning, as the odour presented during the initial habituation trials must be learned for subjects to determine that the second odour is different. Although it has been used to demonstrate mice are able to discriminate a wide range of subtle genetic and metabolic differences between individuals (e.g. Singh *et al.,* 1990; Shellinck *et al.,* 1995; Carroll *et al.,* 2002), this simple test of discrimination provides no further information about the importance of the olfactory signals individuals investigate and is complicated by the issue of familiarity with the first scent presented (Thom and Hurst, 2004). Understanding important processes of recognition and discrimination between individuals regardless of familiarity therefore requires assessment of the behavioural response to olfactory signals beyond simple sniffing and investigation.

Studying the responses of females to male competitive scent marking or male-odour induced pregnancy block provides an opportunity to assess learning of individual scent signatures and recognition more conclusively. For example, following exposure to two scent-marked male territories, females prefer the male owner of a territory in which the male has marked exclusively or has countermarked all intruder scents over a male whose territory has been countermarked by intruders (Rich and Hurst, 1998, 1999). This test of female discrimination and individual recognition overcomes any issues regarding unequal familiarity with the scents, as both intruder marks and countermarks are of equal familiarity to females. The results suggest females are able to recognise the

individual scent owners, and that this is independent of any differences in familiarity with the scents.

Another well studied aspect of natural olfactory learning in mice is the Bruce effect (Bruce, 1959). This effect occurs in newly mated female mice; when exposed to odour from males other than the sire, a high rate of pregnancy failure occurs. This is thought to be adaptive, providing females with an additional opportunity to breed with a male of higher quality or to reduce the risk of infanticide (Schwagmeyer, 1979; Huck, 1984). The mechanism underlying pregnancy block encompasses the learning and memory formation of the scent of the stud male, followed by recognition of this scent and therefore discrimination between other male scents and the scent of the stud. When studied in a laboratory setting, approximately 30% of pregnancies are maintained following exposure to male odour that is not from the original mate (Bruce, 1961). This is significantly lower than the 90% success rate when females are exposed to odours from the sire. Olfactory learning and the memory of a male's odour appears to be contingent upon a prolonged exposure to the mate; females learn the scent signature of the stud male during the first 4-6h after mating (Brennan *et al.,* 1990). In addition, pregnancy disruption is mediated through activation of a vomeronasal neuroendocrine pathway that prevents the release of prolactin, a hormone essential for sustaining early pregnancy (Brennan and Binns, 2005; Stormshak *et al.,* 1987). Activation of this pathway occurs via recognition of a number of male pheromones and MHC-odour based cues of the familiar stud male (Brennan and Peele, 2003); despite being important for individual recognition in mice (Hurst *et al.,* 2001), MUPs appear not to be involved in recognition of familiarity in the context of pregnancy block (Peele *et al.,* 2003). Laboratory studies typically define familiar and unfamiliar males as those of the same strain as the stud male or those of a different strain respectively (e.g. Yamazaki *et al.,* 1983; Peele *et al.,* 2003), although in wild mice, exposure to unfamiliar individuals does not always cause pregnancy block (Coopersmith and Lenington, 1998).

Whilst these two methods for measuring olfactory learning provide a reliable paradigm for quantifying discriminability and individual recognition, they do not provide evidence for how females may assess and respond to male scents, nor do they include assessment of simultaneous spatial learning. Simple tests of attraction to a scent (i.e. increased time spent at the site of the scent) could be used to assess whether females prefer one scent over another, but only olfactory assessment and recognition of

differences between the scents would be measured. To establish whether combined olfactory assessment and spatial learning occurs in female mice in response to male scents, a test of associative learning between a scent and its location would be needed; in this context, the conditioned place preference test is useful.

The conditioned place preference (CPP) paradigm provides a convenient test of how cues can come to support contextual conditioning. In its very basic form it tests the learning of an association between a rewarding stimulus and its presentation in a certain location, thus exploring the combined processing of rewarding cues and spatial learning (reviewed by Tzschentke, 2007), based on classical or associative learning. An event or stimulus considered rewarding is one that has been classically defined as eliciting approach behaviour, thus, if an animal subsequently approaches a stimulus after a prior encounter, it can be considered rewarding (Schneirla, 1959). More recently, reward has been considered to comprise several important components linking a neurological response with behaviour (Berridge and Robinson, 2003). First, for stimuli to be considered rewarding, they must have a positive or pleasurable impact on the animal that is independent of previous experience. Secondly, detection of a stimulus considered rewarding must elicit an immediate and rapid increase in motivation to respond. Finally, a stimulus must be able to induce associative learning for it to be considered rewarding. These properties of rewarding stimuli have been harnessed and utilised in CPP tests that were originally developed to investigate the rewarding or aversive nature of drugs: the context in which the drug is experienced becomes secondarily rewarding or aversive and the animal chooses to spend more time there or elsewhere respectively (reviewed by Carlezon Jr., 2003).

CPP has since been used to investigate the contextual conditioning supported by a wide range of more natural stimuli produced, including conspecific odours and sexual behaviour (Oldenburger *et al.,* 1992; Paredes and Alonso, 1997; Fitchett *et al.,* 2006; Tenk *et al.,* 2009). Results of these experiments show that many socially important cues can be rewarding. For example, previous work by Martinez-Ricos *et al* (2007) shows that female mice form a conditioned place preference for where they have encountered male odours following three presentations of the same odour in a single location. This suggests that male odours constitute a rewarding stimulus for females. Similarly, sexual interactions with males that occur repeatedly in the same location condition a preference for that location in female golden hamsters (*Mesocricetus auratus*),

showing that sexual contact and behaviour is rewarding enough to stimulate a conditioned place preference (Meisel and Joppa, 1994); male hamsters display a similar response (Bell *et al.,* 2010). Soiled nesting material and bedding collected from the home cages of mice can also induce a learned preference for their location in males (Fitchett *et al.* 2006).

In a typical conditioned place preference test commonly used in pharmacological research to measure the rewarding properties of drugs, individual subjects are placed into a test arena containing two distinctly labelled sides. Subjects are first placed into the arena for a 'pre-conditioning' phase, during which time their preference for a particular side of the arena is measured. Repeated presentation of the reward stimulus such as a drug or sugar solution then occurs in the non-preferred side of the test arena; this is considered to be the conditioning phase. The amount of time animals spend on this side is then recorded. Over time the presentation of the reward becomes associated with the location through conditioned learning. Alternatively, sequential confinement is used during the conditioning phase. In this method, animals are confined to their 'non-preferred' side of the test arena (as assessed during pre-conditioning) during administration or presentation of the drug or stimulus being tested. During the next conditioning session, animals are confined to the opposite side of the arena and a control stimulus presented. This confinement procedure is alternated daily and repeated until conditioning is considered complete; the test stimulus is always presented to subjects when confined on their 'non-preferred' side and a control stimulus is always presented when subjects are confined to their 'preferred' side. This method is commonly used as it is thought to control for neophobic responses of animals or the influence of movement and investigatory behaviour during conditioning. The final 'test' phase occurs when the rewarding stimulus is removed and the animal is placed back into the arena and allowed to freely explore both sides; the time spent on the side of the arena where the reward was previously presented is measured. This time is then compared with the original amount of time subjects spent on the same, previously 'non-preferred' side of the arena during the pre-conditioning phase; the difference is taken as a measure of the strength of a conditioned place preference.

Whilst this methodology is useful for measuring the conditioning of a preference for a particular location, and could therefore be useful for measuring female preferences for male scent locations, two main problems are associated with this

standardised procedure. First, comparing the time spent in the conditioned location before and after conditioning does not accurately quantify whether subjects prefer the conditioned location over the non-conditioned location. Rather, it quantifies what is deemed to be a reversal in preference as a result of repeated presentation of the rewarding stimuli. Secondly, the side of the arena that animals spend the least amount of time in during the single pre-conditioning test may simply reflect an arbitrary difference in time spent in each of the two sides and not an actual preference for one side of the arena. As reversal of this 'preference' is often the aim of conditioning in classic tests, designating one side of the arena as preferred based on a single pre-conditioning trial is unlikely to be accurate. It would therefore be important to compare the difference in time spent between the two sides of the arena at the single point in time during the 'test' session when the conditioned stimulus is no longer present.

Despite the potential of the CPP paradigm to measure combined olfactory and spatial learning in the context of female assessment of male scent signals, multiple-trial learning is unlikely to accurately represent olfactory and spatial learning of odour cues in a natural context. Single-trial learning is less common in reward-learning research using conditioned place preference, but several studies have shown that a preference for a location can be induced through a single presentation of a stimulus (e.g. Bardo and Neisewander, 1986; Spina *et al.,* 2006; Roberts *et al.,* 2012). In the natural environment a female may only encounter a male scent mark once before subsequently meeting that male. Acquiring a memory of that location and scent signature after a single learning event would therefore be useful. In addition, females would not encounter single scent marks in the environment, but investigate the scents of multiple males in multiple locations as they move around territories of neighbouring and competing individuals. It is therefore important to address how females respond to multiple scents when presented simultaneously. This would allow assessment of how females make choices between male scents and what factors are important in determining which scents are preferred, whilst simultaneously evaluating how this affects spatial learning of scent locations.

3.1.7 Aims and objectives

Females are attracted to spend time near male scents and can learn the associated volatile signature of an individual scent once contact has been made with that scent (Ramm *et al.,* 2008). Male scent can also condition a learned preference for its remembered location, mediated by the male-specific MUP, darcin (Roberts *et al.,* 2012). Males invest in competitive marking within their territory and at territorial boundaries, exposing females to numerous scent marks from competing males. These reliable indicators of competitive ability and male quality are therefore important for female assessment of males. Acquiring memories of individual scent mark locations is likely to be important in allowing females to locate a preferred male when ready to mate. The overall aims of this study were:

A. To investigate learned spatial preferences of female laboratory mice for single conspecific scents, and to confirm that the same scents and scent components that stimulate learned spatial preferences in wild mice also stimulate this response in laboratory females. This was achieved by presenting a single scent stimulus to female subjects in conditioned place preference tests comprising two locations (scent stimulus and control).
B. To assess how the comparative composition of individual scents influences female learned preferences for locations scent marked by multiple competing males. This was achieved using conditioned place preference tests comprising four locations that allowed multiple scents to be simultaneously presented to females in a competitive context.

In all conditioned place preference tests, after confirming no location bias (no urine), female subjects were presented with the scent stimulus or stimuli and control(s) during a single, 10 minute learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). To achieve the above aims, the individual objectives of this study were to answer the following questions:

1. **Are learned spatial preferences of female laboratory mice for the location of a scent influenced by the sex or strain of the scent donor?**

   Females show a learned preference for the location of male odour when it is presented on multiple occasions in the same location (Martinez-Ricos *et al*, 2007); this learned spatial preference can be conditioned in wild mice using a single

presentation of male urine alone (Roberts *et al*, 2012). To confirm that the scent that conditions place preference in laboratory mice is in male urine, female subjects were presented with a single male or female urine stimulus collected from donors of their own strain (CD-1). As presenting subjects with urine collected from donors of their own strain raises the potential issue of prior familiarity, to assess if a learned preference for a urine location is a generalised response to male urine or if novel urine regardless of sex can condition a preference, two additional groups of females were presented with either male or female urine collected from a second, novel strain (C57BL/6).

2. **Is the male pheromone, darcin, required for male scent to condition a place preference for its location?**

   This objective was to confirm that the male pheromone, darcin, is important for conditioned place preference in female laboratory mice. To test this, females were presented with a single urine stimulus, either male BALB/c urine, which contains negligible amounts of naturally produced darcin (Cheetham *et al.,* 2009; Roberts *et al.,* 2010), or male BALB/c urine with the addition of 1μg r-darcin per 1μl of urine, a concentration roughly equivalent to that naturally expressed in the urine of C57BL/6 males (10-14% of overall MUP expression) (Armstrong *et al.,* 2005).

3. **Can females form learned spatial preferences for multiple scents?**

   In a natural context, females may explore areas encompassing the territories of several different males. Assessing competing male scents and acquiring memories of preferred scent mark locations is likely to be important in allowing females to locate a preferred male when ready to mate. The next objective of this study was to determine whether females can form learned preferences for multiple male scents presented simultaneously. Female subjects were presented with two equivalent male urine stimuli (CD-1; the subjects' own strain) and two identical control stimuli (ddH_2O) during the learning session.

4. **Is the male specific pheromone, darcin, important in female learned preferences for multiple male scent locations?**

   To assess the importance of this male specific pheromone in the induction of female attraction and learned spatial preferences for multiple male scents, two questions were addressed:

   a. Is the presence of darcin required in all competing male scents for them to both stimulate a learned a preference in females?

b. When presented with multiple male scents that contain equivalent concentrations of darcin, do females respond differently according to whether darcin is naturally produced or added to male urine as a recombinant protein?

To answer the first of these questions, a test comprising four stimuli locations was used; female subjects were presented with the two different urine stimuli, male BALB/c urine (containing negligible amounts of naturally produced darcin) and male C57BL/6 urine (containing approximately 1μg/μl darcin) during the learning session. To address the second question, a separate place preference test was conducted. In this test, females were presented with two male urine stimuli containing equivalent amounts of either naturally produced darcin or recombinant darcin and two control stimuli (ddH_2O).

5. **Are female learned preferences for male urine with added recombinant darcin specifically due to the addition of darcin?**

   Addition of recombinant darcin to male BALB/c urine increases the overall concentration of protein in urine as well as increasing the concentration of darcin specifically. The learned spatial preference of females for locations of male BALB/c urine with the addition of r-darcin may therefore be influenced by this increase in overall protein concentration. To ensure that females respond to the presence of darcin specifically and not an overall increase in protein, female subjects were presented with male BALB/c urine with the addition of r-darcin or equivalent concentrations of other r-MUPs during the learning session.

6. **Can darcin stimulate a learned preference for its location?**

   The male specific MUP, darcin, stimulates a learned preference in wild female mice for its location (Roberts *et al.,* 2012). To establish whether this involatile pheromone alone can condition a learned preference in female laboratory mice for its location, female subjects were presented with r-darcin or other recombinant MUPs and a single control stimulus (phosphate buffer) during the learning session.

7. **What is the minimum concentration of darcin required for male scent to condition a preference for its location?**

   The presence of darcin in male urine is essential for females to form a learned preference for the location of male scent. This suggests investment in darcin within scent marks is an important aspect of male scent signalling. However, it is unknown

what minimum concentration of darcin is required to stimulate female learning, and how this compares to normal levels of investment by wild males. To investigate what concentration of darcin must be present in male urine for it to induce a learned spatial preference for its location, female subjects were exposed to male BALB/c urine containing a range of concentrations of r-darcin as low as 0.025μg/μl (2.5% of the concentration known to reliably stimulate a learned preferences).

8. **How does the estimated threshold concentration compare to normal darcin expression among wild male mice?**

   To determine how the estimated threshold concentration assessed from conditioned place preferences tests compares to the natural expression levels of wild male mice, urinary protein and darcin concentration was measured in urine samples collected from 40 wild stock males bred in the laboratory.

9. **Do differences in darcin concentration between male scents influence female learned preferences for competing male scents?**

   Adult male mice consistently express high levels darcin in their urine and under competitive conditions males increase their investment in MUPs (Garratt *et al.,* 2012). The amount of darcin present in a male scent mark may therefore be important for female assessment. The next objective of this study was to assess how differences in darcin concentration between multiple male scents influences learned spatial preferences of females. Using two separate conditioned place preference tests, each comprising four locations, female subjects were presented with male BALB/c urine with the addition of different concentrations of r-darcin and a single control stimulus (ddH_2O) during the learning session. In each test, females were presented with male urine containing a 'low' concentration of darcin and male urine containing a 'high' concentration of darcin; the influence of the magnitude of the difference between these scents was also assessed. Analysis of female responses to these scents allowed assessment of whether females respond differently to scents that contain different concentrations of darcin, and if the magnitude of the difference in darcin concentration between the scents is important.

10. **Does the age of a male scent influence female learned preferences?**

    Females continue to show an attraction to male urine that has been aged for up to 7 days (Roberts *et al.,* 2010), even though 98% of the volatile pheromone this protein binds, thiazole, may be lost during the first 24 hours after a scent is deposited (Armstrong *et al.,* 2005). The age of male scent therefore influences its composition,

potentially affecting whether it is able to induce a learned preference for its location. To assess whether the age of a scent influences learned spatial preferences in laboratory mice, females were presented with either fresh male urine, or male urine that had been aged for 7 days. Using a test comprising two locations, females were presented with fresh or aged stimulus urine and a control stimulus (ddH_2O) during the learning session.

11. **Does scent age influence female learned preferences for competing male scents that differ in age?**

    Male mice invest in competitive countermarking when intruders deposit scent marks within their territory and constantly refresh these marks to reinforce their dominance (Hurst, 1993); scent freshness indicates the ability of a male to effectively defend his territory. A remembered preference for the location of the freshest of multiple scents may increase the likelihood that a female will return to the territory of a male of high competitive ability. As a result, scent age may be important for learned preferences in a competitive context. The role of scent age in a competitive context was addressed using a conditioned place preference test where fresh and aged male urine were presented simultaneously to females.

12. **Does familiarity influence learned spatial preferences for male scent locations?**

    Females find the owner of familiar scents more attractive than scents of completely novel individuals (Cheetham, 2006). This preference may be beneficial as assessment of long term male quality in terms of health status and competitive ability may be more accurate if a male or his scent has been encountered several times. To assess the importance of prior familiarity on learned spatial preferences for male scent locations, urine collected from individual wild stock males was used in a conditioned place preference test comprising four locations. Unlike laboratory mice, which exhibit uniform expression of MUPs across individuals of each strain (Cheetham *et al.,* 2009), individual wild mice each express a unique MUP profile, providing a stable and reliable signature of individual identity (Hurst *et al.,* 2001). Prior to the learning session, females were exposed to urine from one individual wild stock male for 30 minutes; this urine was then considered familiar to subjects. Female subjects were then presented with stimulus urine (familiar urine and unfamiliar urine from a second, wild stock male donor) and two control stimuli (ddH_2O) during the learning session.

3.2 METHODS

3.2.1 Subject animals

Subject females (*n* = 150) were CD-1 laboratory mice (Harlan, Loughborough, UK) aged between 4 and 12 months. Females were either obtained from an approved supplier (Harlan, UK) at 3-4 weeks of age or bred in house (parents obtained from Harlan, UK). At 3-4 weeks mice were house in either same-sex pairs in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK) or in same-sex groups of four in 45cm x 28cm x 13cm cages (MB1, North Kent Plastics Ltd., UK); pairs and groups were maintained throughout the study. Throughout the experiment all animals were maintained on a reversed 12:12 light cycle (lights off at 08:00h), housed on Corn Cob Absorb 10/14 substrate with paper wool bedding material and given *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, MO, USA). Perspex tunnels (Techniplast, NJ, USA) were provided for home cage enrichment. All subjects were used in at least one test; some were used in more than one test but always with different unfamiliar test stimuli and a minimum of 3 weeks between successive tests (36/150 (24%) used twice, 30/150 (20%) used three times, 12/150 (8%) used four times.).

3.2.2 Urine donors and urine collection

Subject females also served as urine donors for other subject females. Adult male (n = 48) and adult female (n = 12) C57BL/6JOlaHsd laboratory mice, adult male (n = 24) CD-1 laboratory mice, adult male (n = 48) and adult female (n = 12) BALB/c laboratory mice, and adult male (n = 32) wild stock mice provided urine for tests. All animals used for urine collection were 6 - 12 months old and were either obtained from an approved supplier (Harlan, UK) at 3-4 weeks of age or bred in house (parents obtained from Harlan, UK). At 3-4 weeks mice were housed in same-sex pairs in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK) or in same-sex groups of four in 45cm x 28cm x 13cm cages (MB1, North Kent Plastics Ltd., UK); pairs and groups were maintained throughout the study but on rare occasions where aggression was observed between paired or grouped males individuals were separated and housed singly in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK). Throughout the experiment all animals were maintained on a reversed 12:12 light cycle (lights off at

08:00h) and on Corn Cob Absorb 10/14 substrate with paper wool bedding material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, MO, USA). Perspex tunnels (Techniplast, NJ, USA) were provided for home cage enrichment.

Urine was collected from laboratory mouse donors by holding the mouse by the scruff of the neck over a clean 1.5ml Eppendorf tube. Urine samples were collected from wild-stock mice by confining males above a cage on a mesh grid for a maximum of 2h. Cages under males were checked every 30 min and if urine was present it was collected immediately and stored at -20°C. Laboratory mice exhibit uniform expression of the urinary MUP olfactory identity signature across individuals of each strain (Cheetham *et al.,* 2009), so urine from up to 8 donors of the same strain and sex was pooled for testing using different combinations of donors to create each pool. Multiple pools provided stimuli for an equal number of replicates when used in the same test. For example, if three separate pools provided a sufficient volume of urine for use as stimuli in a test comprising twelve female subjects, urine from each pool was presented as the stimulus to four females. By contrast, urine collected from wild-stock males was not pooled as each individual excretes a different urinary identity signature. All urine was collected up to 1 week prior to testing and stored at -20°C until use.

3.2.3 Conditioned place preference tests

Conditioned place preference tests comprising two locations were conducted following a method similar to that described in Roberts *et al.,* (2012). Tests were conducted in clean 45cm x 28cm x 13cm arenas (MB1 cage base fitted with a perforated clear plastic lid) containing two different 14.5cm x 14.5cm plastic tiles to provide internal spatial cues: one black plastic tile on the right hand side and one clear plastic tile with black edging on the left hand side (**Figure 3.2a**). Plastic tiles were stuck down to the floor of the arena with reusable adhesive (Blu Tack, Bostik Limited, UK) approximately 4cm apart and 2.5cm from each side. Plastic petri dishes (55mm diameter) containing 55mm diameter glass microfibre filter papers (Whatman, grade GF/C) were stuck down on the centre of each tile with double sided adhesive tape. The petri dishes were approximately 12cm apart.

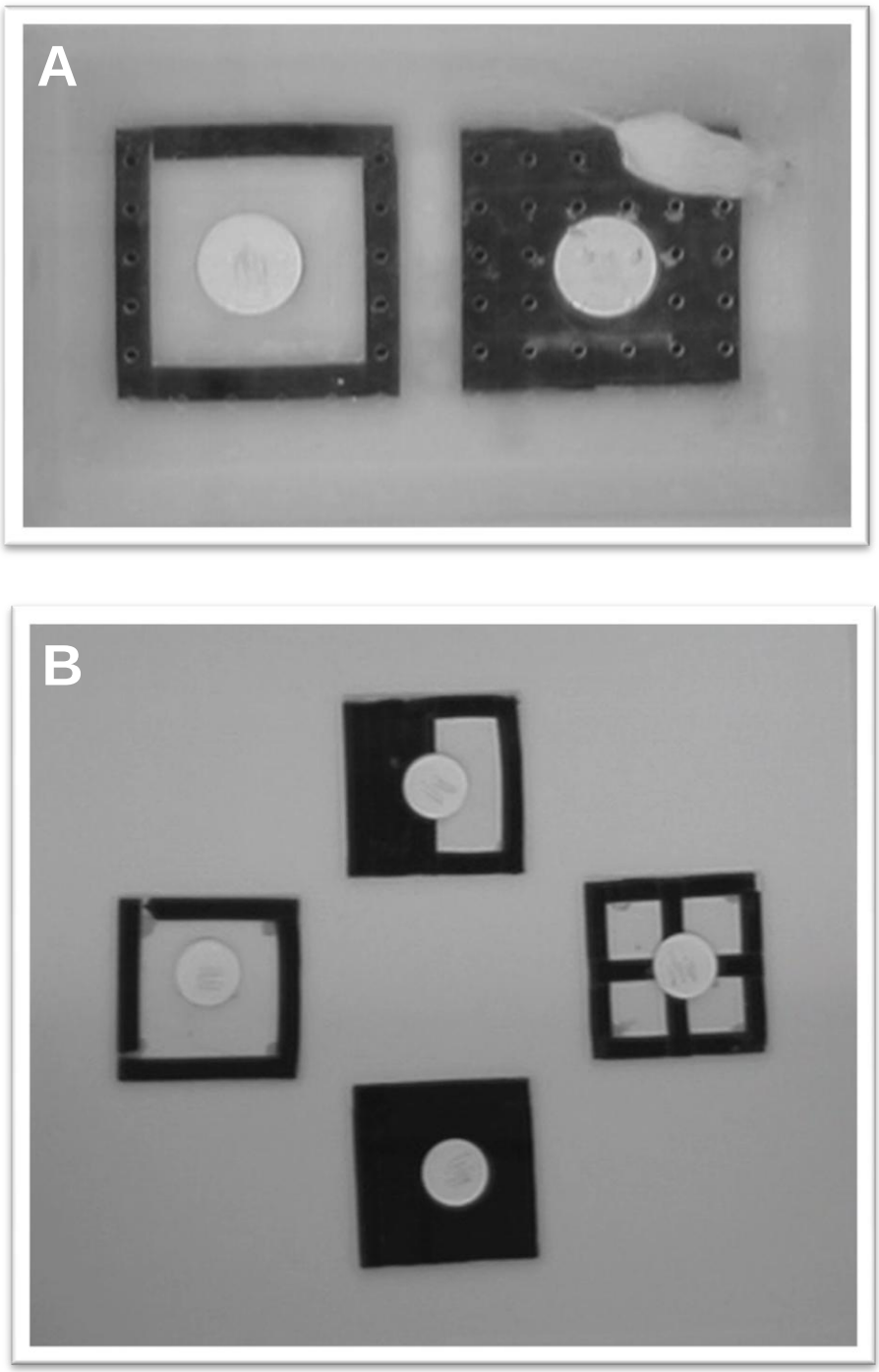

Figure 3.2 Test arenas for trials using (a) 2 locations or (b) 4 locations.

Conditioned place preference tests comprising four locations were conducted in a laminated MDF arena measuring 70cm x 60cm x 55cm, containing four different 14.5cm x 14.5cm tiles to provide internal spatial cues: one black plastic tile on the near-side, one clear plastic tile with black edging on the left hand side, one half clear, half black plastic tile on the far-side and one clear plastic tile with a black cross on the right hand side (**Figure 3.2b**). The four tiles were fixed to the base of the arena at the midpoint along each edge approximately 6cm from the outer wall with reusable adhesive (Blu Tack, Bostik Limited, UK). Plastic petri dishes (55mm diameter) containing 55mm diameter glass microfibre filter papers (Whatman, grade GF/C) were stuck down on the centre of each tile with double sided adhesive tape. The petri dishes were between 22cm and 37cm apart.

In all tests additional external cues were provided by the consistent locations of overhead red lighting in the room and a radio placed in a constant location; the radio provided even background noise typical of the level heard during daily cage cleaning and animal handling procedures. All tests took place during the dark phase of the light cycle. Animals were handled using clear acrylic home cage tunnels that were present in the cages of all subjects. A tunnel was brought towards the animal and held resting on the cage substrate before guiding the animal towards the tunnel with the other hand. Hands were cupped loosely over the tunnel ends to prevent escape and the tunnel lifted out of the home cage and lowered as slowly and gently as possible into the test arena. Hands were then removed from the tunnel ends and the animal allowed to exit. In cases where subjects were reluctant to exit the tunnel it was tipped at a slight angle to allow the animal to slowly slide backwards out of the tunnel. This method of handling was chosen as an alternative to standard handling of mice by the tail to minimise aversion and anxiety that may influence variation in behaviour (Hurst and West, 2010).

All conditioned place preference tests, regardless of the arena used, consisted of three stages conducted across three days. During all stages, internal and external location cues remained consistent and the arena was thoroughly cleaned with 70% ethanol and allowed to dry between trials. During stage one, subjects were placed into the test arena for an initial 10min habituation period to confirm the absence of a pre-existing location bias and to familiarise subjects to the test arena. During habituation, all petri dishes (two or four depending on the number of locations in the test) contained 50µl ddH_2O on the filter paper as 5 x 10µl streaks. Stage two consisted of a 10 minutes

learning session and took place 24 hours after stage one. During this learning session, in conditioned place preference tests comprising two locations, 50µl of a urine stimulus was presented in one of petri dishes as 5 x 10µl streaks to mimic scent marks; in tests with four locations between one and four different urine stimuli were presented. 50µl ddH_2O was presented in the remaining petri dish, or remaining dishes in tests with four locations, serving as a control stimulus. The position of the urine stimulus or stimuli was randomised between subjects but balanced as far as possible to ensure that the stimulus or stimuli were presented in each location a similar number of times. Stage three, the conditioned place preference test, took place 24 hours after the learning session and lasted 10min. This stage was a repeat of stage one; 50µl ddH_2O control stimulus was presented in each of the petri dishes. Between all trials petri dishes and filter papers were removed and not reused.

When assessing the response of females to r-MUPs alone, 50µL of the recombinant MUP in 50mM phosphate, 20mM NaCl pH7.4 was used as the test stimulus. In these tests, 50µL of the same buffer replaced ddH_2O as the control stimulus. The r-MUPs used tests were male-specific r-MUP7 (molecular weight 18645Da) and non-sex specific r-MUP11 (molecular weight 18694Da). When assessing the response of females to r-MUPs added to male urine, r-MUPs were added at a concentration of 10µg to 10µl male urine (unless otherwise stated) to mimic the natural concentration of darcin observed in C57BL/6 and wild males (approximately 10-14% of total MUP) (Roberts *et al.,* 2010). To ensure that manipulated urine and control stimulus were treated equally, an equivalent volume of 50mM phosphate, 20mM NaCl buffer, pH7.4 was added to the corresponding control stimulus. In tests where familiarity with the scent was assessed, wild male urine was used to provide scent stimuli with distinct olfactory signatures. Subjects were pre-exposed to 50µl of urine from an individual male in a clean 45cm x 28cm x 13cm cage (MB1, North Kent Plastics Ltd., UK) for 30 minutes immediately prior to the learning session. Subjects were always able to make direct contact with the scent during pre-exposure.

To reduce variability in a factor that may potentially influence the response of females, the oestrus stage of subjects was manipulated. Soiled nesting material from males was introduced into the cages of subjects 3 days prior to the learning phase of the trials. This enhanced the likelihood that all females would be in proestrus or oestrus on the learning day (Cheetham *et al*, 2007). The behaviour of subjects in each of the trials

was recorded remotely on DVD for all stages of tests. DVD recordings were then transcribed blind relative to the location of the different stimuli. An event recording program (written by Prof. R Beynon, Protein Function Group, University of Liverpool) was used to record time spent sniffing the dish (head in the dish and nose making sniffing movements) and time spent on the tile around the dish (all four paws on the tile). This data was subsequently decoded using an SPSS syntax (written by Prof. J Hurst, Mammalian Behaviour and Evolution Group, University of Liverpool), which translates information recorded by the event recording program to provide the total time females spent on each tile around the petri dishes and time spent sniffing in each of the dishes.

In conditioned place preference tests conducted by Roberts *et al.,* (2012), an additional variable of time spent in each petri dish (whole body or head inside the dish) was recorded. However, female subjects used in the tests outlined here were typically too large to fit comfortably inside the petri dishes and the majority of females failed to spend any time sitting in any of the dishes. Time spent sniffing and time on the tile around the dish were therefore combined to create a "total time in stimulus location" variable, which was the best measure of attraction during the learning sessions indicated by initial responses of female subjects to male urine. This variable was used in all subsequent data analyses.

3.2.4 Measurement of urinary protein and creatinine concentration

Protein concentrations were determined using the Coomassie Plus protein assay reagent kit from Perbio Science UK Ltd. (Northumberland, UK) following methods described by Cheetham *et al.* (2009) and discussed previously in section 2.2.5. Urinary creatinine values were measured by the alkaline picrate assay from Sigma Chemicals, UK as described in section 2.2.5.

3.2.5 Measurement of urinary darcin concentration

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide) was performed as described by Laemmli (1970) and discussed previously in section 2.4.6.

3.2.6 Expression and purification of recombinant MUPs

Recombinant MUPs were expressed and purified by Lynn McLean (Protein Function Group, University of Liverpool, UK) using methods described by Roberts *et al*, 2010. The primary sequence of darcin was obtained from the sequence located under accession numbers NP_001012323/XP_355497 and was used to direct *de novo* gene synthesis for maximal expression in *E. coli*. The gene was codon optimised for expression in *E. coli* and cloned into pET28b via *Nco*I and *Xho*I restriction sites (Entelechon GmBH, Regensburg, Germany). The plasmid was used to transform BL21(λ)DE3 cells and darcin expressed in Luria Broth containing kanamycin (30μg/mL). At OD600nm of between 0.6-0.8, the expression of recombinant darcin (r-darcin) was induced by the addition of isopropyl β-D-1 thiogalactopyranoside to a final concentration of 1 mM. Five hours post-induction, cells were harvested by centrifugation at 2000g and the cell pellets stored at -20°C prior to further purification. Harvested cells were lysed using Bugbuster protein extraction reagent (Novagen, Nottingham, UK) containing Complete™ EDTA-free protease inhibitor cocktail (Roche, Burgess Hill, UK). Darcin, present in the soluble fraction of the bacterial cell lysate was purified by virtue of the hexahistidine tag on nickel affinity columns according to manufacturer's protocols (Novagen). Column fractions containing r-darcin were pooled and dialysed against 50mM phosphate, 20mM NaCl pH7.4. This preparation was used without further processing. The purity of r-darcin was assessed by SDS-PAGE analysis and protein concentration was determined by protein assay. Similar workflows were applied to express the 18645Da MUP (accession GB/AAH91744.1) and 18694Da MUP (accession NP_001157998.1). Recombinant darcin and other recombinant MUPs were tested alone (10μg in 10μL buffer) or added to male urine at the concentration specified in each particular test.

3.2.7 Data Analysis

Females were expected to show significantly greater attraction to a scent stimulus compared to a water or buffer control during learning sessions and to spend more time in the learned location of a scent stimulus if the scent stimulated a conditioned place preference. For all tests, the difference between time spent in the location where the scent stimulus was presented during the learning session and the

time spent in the location of the control stimulus was analysed; in tests comprising more than one control location, the mean time spent in the control locations was used in the analysis. Where data distribution approximated normality (assessed by Kolmogorov Smirnov and Shapiro Wilks tests $p > 0.05$), whether females spent significantly longer in the location of a scent stimulus compared to the location of the control stimulus was assessed by matched-pair t-tests (one-tailed to assess greater time in scent stimulus location) or repeated measures ANOVA with planned contrasts comparing time in scent location versus control location. Independent Student's t-tests compared the strength of biases (time in scent stimulus location minus control location, or difference in time spent in locations of two specific scents) between specific tests. Where data could not be transformed to approximate normality, non-parametric Wilcoxon signed ranks tests or Friedman tests assessed greater time in the location of the scent stimulus compared to the location of the control; Mann-Whitney U tests compared the strength of biases between specific tests. All trials were initially conducted with $n = 12$ female subjects (occasionally tests were eliminated when mice showed stereotypical behaviour or failed to visit the location of the scent stimulus during the learning session). The results of some trials approached significance, so to increase statistical power and confirm a response the sample size was increased to $n = 16$ or 17.

During the habituation stage of all tests, no significant pre-existing bias for any of the locations was observed, indicating that females used in these tests did not demonstrate a preference for a particular location before scent stimuli were presented. A conditioned place preference test comprising four locations was also conducted with only control stimuli (ddH_2O) presented to female subjects at all stages of the test to confirm that subjects habituate to the test arena during the course of the experiment. When females were presented with four control stimuli (ddH_2O), no significant difference in the time spent in each of the control locations was observed on any of the three test days (repeated measures ANOVA: habituation: $F_{(3,33)} = 1.20$, $p = 0.33$; learning: $F_{(3,33)} = 0.17$, $p = 0.91$; 24h memory: $F_{(3,33)} = 0.46$, $p = 0.72$; **Figure 3.3**). However, the mean time spent on the stimulus tiles declined significantly across the three test days (repeated measures ANOVA: $F_{(2,33)} = 4.10$, $p = 0.03$). This suggests females habituate to the test arena during the three stages of the test. Given this habituation, it is not appropriate to compare female responses during the 24h place

preference test with those recorded during habituation; rather, time spent in each location should be compared during each stage of the test.

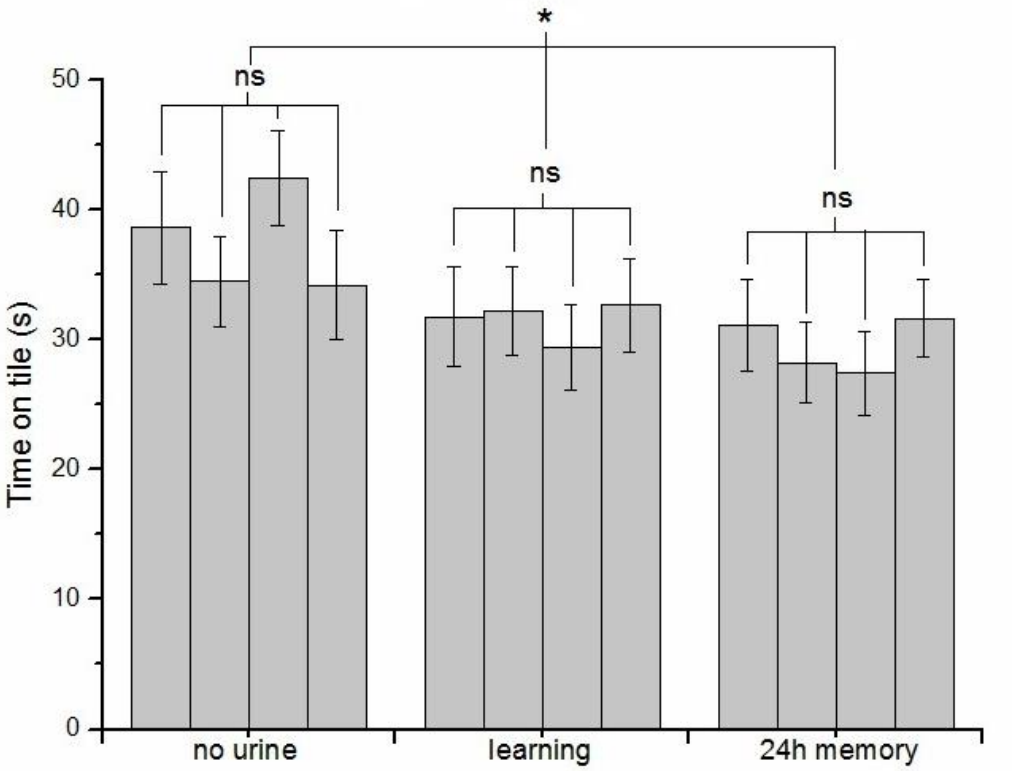

Figure 3.3 Female attraction and learned spatial preferences for four control stimuli (ddH_2O).

Time spent by female subjects in four control locations during three 10-min sessions on consecutive days of a conditioned place preference test (n = 12 subjects). Separate repeated measures ANOVAs tested for significant differences in time spent in each location during each 10-min session and in the mean time spent on tiles across the three days of testing * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3 RESULTS

3.3.1 Are learned spatial preferences of female laboratory mice for the location of a scent influenced by the sex or strain of the scent donor?

Females show a learned preference for the location of male odour when it is presented on multiple occasions in the same location (Martinez-Ricos *et al*, 2007); this learned spatial preference can be conditioned in wild female mice using a single presentation of urine alone (Roberts *et al*, 2012). To confirm that the scent that conditions place preference in laboratory mice is in male urine, female subjects were presented with male or female urine from their own strain (CD-1). Using a test comprising two locations, after confirming no side bias (no urine), female subjects were presented with the stimulus urine (male or female) and the control stimulus (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). In these tests and in previous studies using female laboratory mice as subjects, females were only presented with odours from males or females of their own strain (Martinez-Ricos *et al*, 2007). This raises the potential issue of familiarity, as scents from males and females of the subjects own strain would be encountered prior to weaning and as a result of being caged with same-sex siblings. To assess if associative learning of urine locations is a generalised response to male urine or if novel urine regardless of sex can condition a preference for its location, two additional groups of females were presented with either male or female urine collected from a second, novel strain (C57BL/6). In this first experiment, females were presented with male CD-1 urine, female CD-1 urine, male C57BL/6 urine or female C57BL/6 urine to examine the influence of donor strain and sex on female learned preferences for scent locations.

Female attraction to urine during the learning session differed significantly according to the sex of the urine stimulus (interaction between stimulus and sex: $F_{(1,44)} = 8.55$, $p = 0.005$), but did not differ according to the strain of the urine (interaction between stimulus and strain: $F_{(1,44)} = 1.40$, $p = 0.24$). This was due to a lack of attraction to either CD-1 or C57BL/6 female urine (matched pair t-test: CD-1: $t_{11} = 0.12$, $p = 0.91$; C57BL/6: $t_{11} = -0.49$, $p = 0.64$; **Figures 3.4a & b**), and a significant attraction to male urine from both strains (CD-1: $t_{11} = -3.88$, $p = 0.003$; C57BL/6: $t_{11} = -4.79$, $p < 0.001$; **Figures 3.4c & d**).

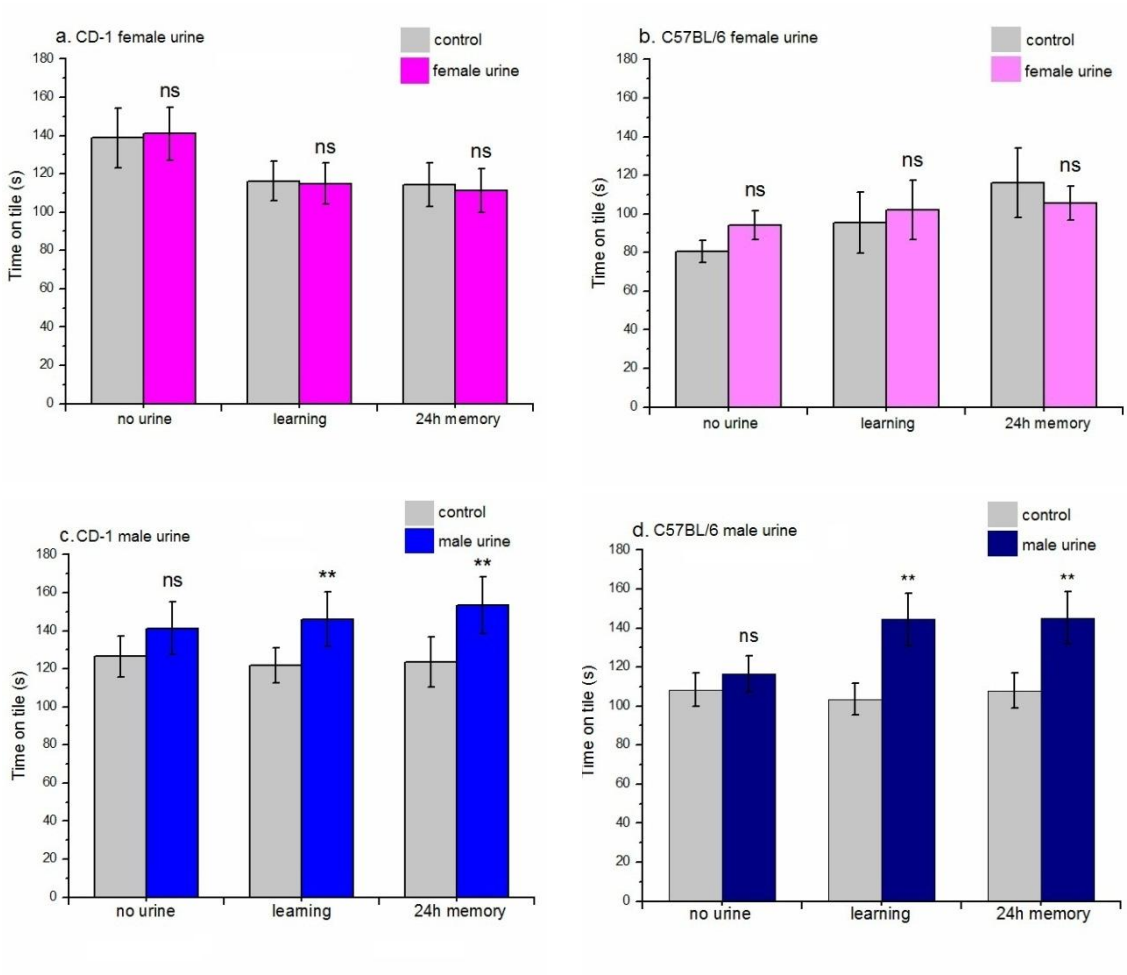

Figure 3.4 Female attraction and learned spatial preferences for locations of male or female urine.

Time spent by female subjects in urine or control locations when presented with (a) female CD-1 urine, (b) female C57BL/6 urine, (c) male CD-1 urine or (d) male C57BL/6 urine. [(a), (b), (c) and (d) $n = 12$ subjects.] P values test for more time in urine versus control location * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

Similarly, female learned preferences differed significantly according to the sex of the urine stimulus (interaction between stimulus and sex: $F_{(1,43)} = 15.82$, $p < 0.001$), but did not differ according to the strain of the urine (interaction between stimulus and strain: $F_{(1,43)} = 0.00$, $p = 0.99$). A learned spatial preference was not observed in females exposed to female urine from either their own strain or a novel one (matched pair t-test: CD-1: $t_{11} = 0.35$, $p = 0.74$; C57BL/6: $t_{10} = 0.87$, $p = 0.41$; **Figures 3.4a & b**), but females did form a learned preference for male urine from either their own or a novel strain (CD-1: $t_{11} = -3.20$, $p = 0.008$; C57BL/6: $t_{11} = -3.84$, $p = 0.003$; **Figures 3.4c & d**). Male, but not female urine induced a significant attraction and learned preference for its location, regardless of whether this urine was from individuals of the same strain as subjects or from a strain novel to subjects.

3.3.2 Is the male pheromone, darcin, required for male scent to condition a place preference for its location?

The male pheromone, darcin, is required for male scent to condition a preference for its location in wild female mice (Roberts *et al*, 2012). The objective of tests outlined below was to confirm that this male pheromone is also important for conditioned place preference in laboratory mice. Due to inbreeding over many generations in the laboratory, sexual selection pressures have been negated; as a result, males of the laboratory strain BALB/c produce negligible amounts of darcin (Cheetham *et al.,* 2009; Roberts *et al.,* 2010). To test whether darcin is important for learned spatial preferences in laboratory mice, females were presented with either male BALB/c urine, which contains negligible amounts of naturally produced darcin, or male BALB/c urine with the addition of 1μg r-darcin per 1μl of urine, a concentration roughly equivalent to that found in the urine of C57BL/6 males (10-14% of overall MUP expression) (Armstrong *et al.,* 2005). Using a test comprising two locations, after confirming no side bias (no urine), female subjects were presented with the stimulus urine (male BALB/c urine or male BALB/c urine + r-darcin) and a control stimulus (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory).

When presented with male BALB/c urine during the learning session, females showed no significant attraction to the location of male urine (Wilcoxon signed ranks

test: Z = -0.16, p = 0.46; **Figure 3.5a**). Further, females did not demonstrate a learned spatial preference for male BALB/c urine (Z = -0.08, p = 0.49; **Figure 3.5a**). By contrast, females presented with male BALB/c urine with the addition of 1μg r-darcin per 1μl urine showed a significant attraction during the learning session and learned spatial preference during the 24h memory test for the location of this male urine (Wilcoxon signed ranks test: learning: Z = -2.35, p = 0.008; 24h memory: Z = -2.59, p = 0.003; **Figure 3.5b**). Female attraction to the location of BALB/c male urine with the addition of 1μg/μl r-darcin was significantly greater than the attraction of females to male BALB/c urine alone during the learning sessions (Mann Whitney U test: U = 43.00, Z = -1.67, p = 0.05). Similarly, the learned spatial preference of females for male BALB/c urine with the addition of r-darcin was significantly different to the response to BALB/c urine alone (U = 42.00, Z = -1.73, p = 0.04). Male BALB/c urine containing negligible levels of naturally produced darcin did not stimulate attraction or condition a learned preference for its location. However, females presented with male BALB/c urine with the addition of 1μg/μl r-darcin showed a significant attraction and learned spatial preference for the location of this male urine. In summary, the male specific pheromone darcin appears to be important for learning of male scent locations in female laboratory mice.

3.3.3 Can females form learned spatial preferences for multiple scents?

In a natural context, males invest in competitive marking within their territory and at territorial boundaries, exposing females to numerous scent marks from competing males. Many of these marks may be close in proximity to each other, particularly at territorial boundaries. Assessing competing male scents and acquiring memories of preferred scent mark locations is likely to be important in allowing females to locate a preferred male when ready to mate. The next of objective of this study was to assess how females respond to multiple male scents presented in a competitive context. To achieve this, conditioned place preference tests comprising up to four scents in four different locations were used.

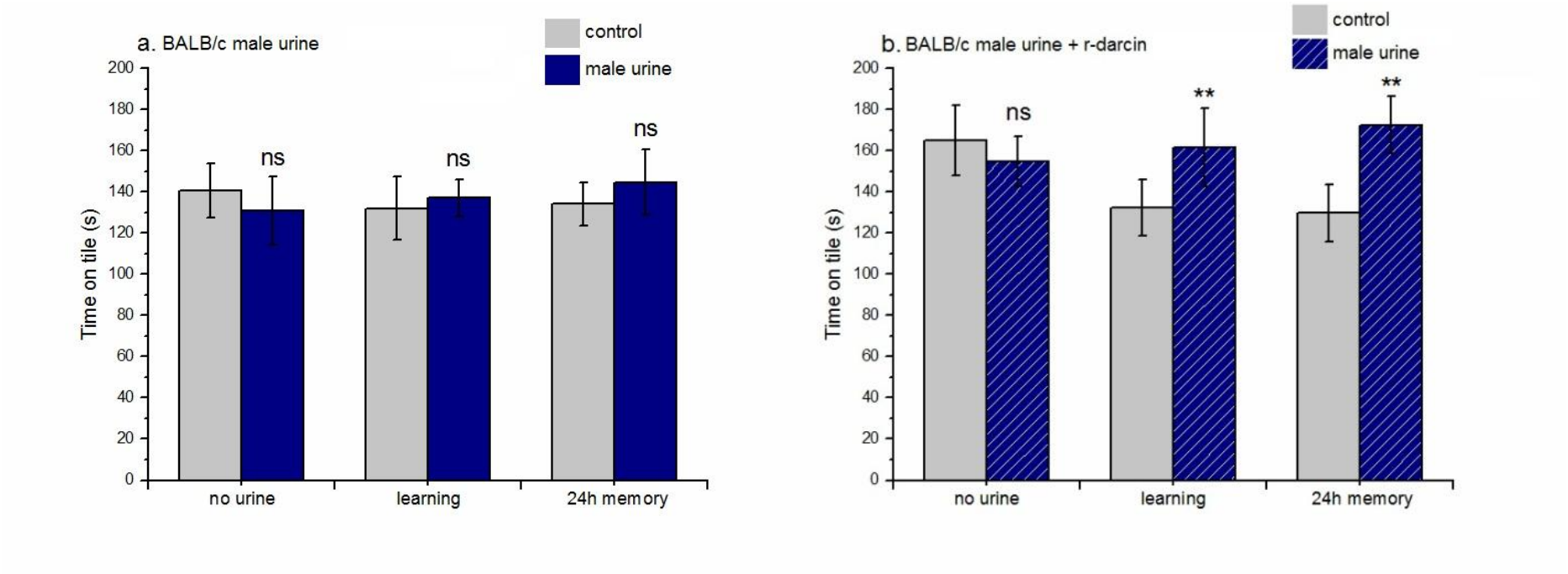

Figure 3.5 Female attraction and learned spatial preferences for male urine containing no darcin or 1µg/µl r-darcin.
Time spent by female subjects in urine or control locations when presented with (a) male BALB/c urine or (b) male BALB/c urine + 1µg/µl r-darcin. [(a) and (b) n = 12 subjects.] P values test for more time in urine versus control location * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

Using a test comprising four locations, after confirming no side bias (no urine), female subjects were presented with two identical control stimuli (ddH_2O) and two equivalent male CD-1 urine stimuli during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). Females showed a significant attraction to both locations of male urine (repeated measures ANOVA: $F_{(2,22)} = 10.18$, $p = 0.001$; **Figure 3.6a**). Further, females demonstrated a learned spatial preference for both male urine locations ($F_{(2,22)} = 13.33$, $p < 0.001$; **Figure 3.6a**). Females formed learned spatial preferences for locations of multiple male scents when these scents were equivalent.

When presented with single male or female urine stimuli females show a significant attraction and learned preference for the location of male, but not female urine. This response occurs regardless of the whether urine is from individuals of the subjects' own strain or a novel strain. To determine whether females demonstrate a significant attraction and learned preference for male, but not female urine when females are exposed to these different scents simultaneously, female subjects were presented with male and female urine from two different, novel strains (male C57BL/6 urine and female BALB/c urine) in a four location conditioned place preference test. During the learning session, there was an overall effect of stimulus on female attraction (repeated measures ANOVA: $F_{(2,22)} = 22.45$, $p < 0.001$); females showed a significant attraction to male, but not female urine (male urine $F_{(1,11)} = 46.91$, $p < 0.001$; female urine: $F_{(1,11)} = 0.91$, $p = 0.36$; **Figure 3.6b**). Similarly, there was an overall effect of stimulus on female learned preferences ($F_{(2,22)} = 9.21$, $p = 0.001$); females demonstrated a learned spatial preference for the location of male, but not female urine (male urine $F_{(1,11)} = 35.44$, $p < 0.001$; female urine: $F_{(1,11)} = 0.02$, $p = 0.90$; **Figure 3.6b**). Male but not female urine induced a significant attraction and learned preferences for its location, even when these scents were presented simultaneously.

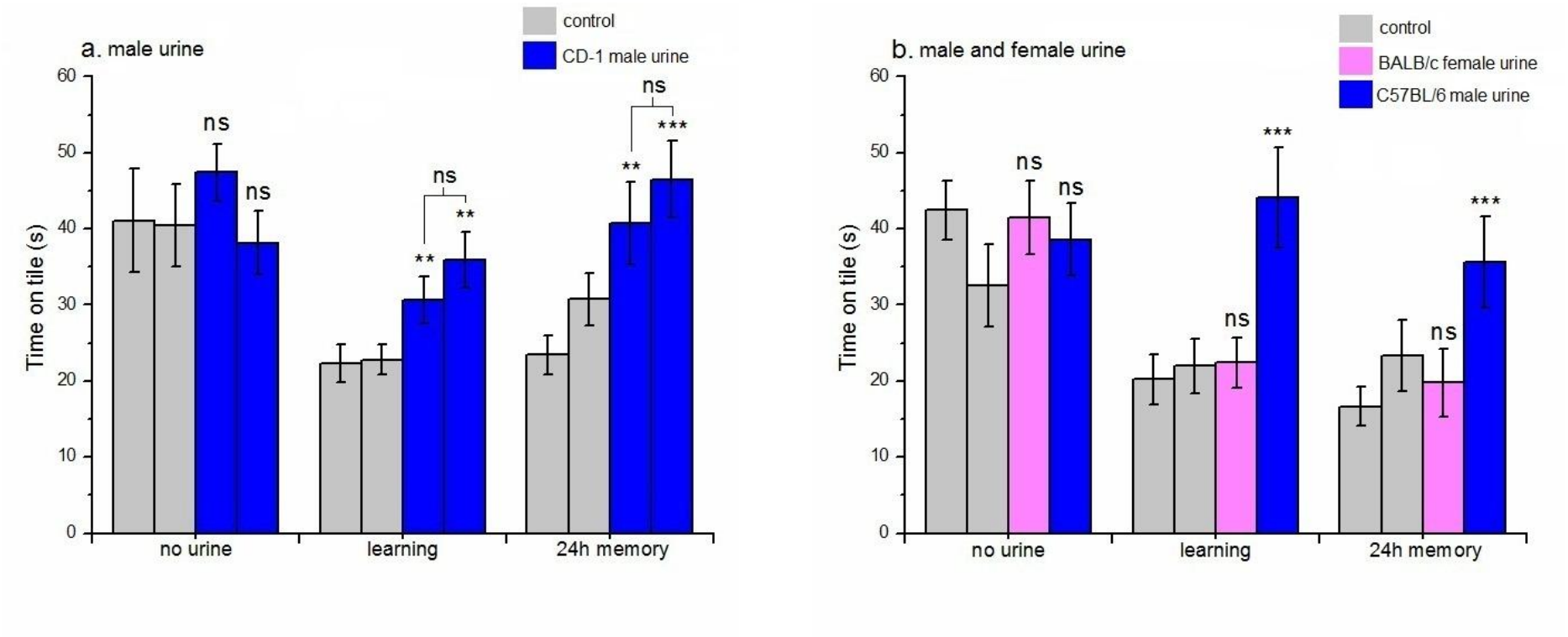

Figure 3.6 Female attraction and learned spatial preferences for multiple urine locations.
Time spent by female subjects in urine or control locations when presented with (a) two male CD-1 urine stimuli or (b) male C57BL/6 urine and female BALB/c urine. [(a) and (b) $n = 12$ subjects.] P values test for more time in urine versus mean time in control locations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3.4 Is the male specific pheromone, darcin, important in female learned preferences for multiple male scent locations?

When presented with a single urine stimulus in conditioned place preferences tests comprising two locations, females only demonstrate a learned spatial preference for the location of male urine if it contains darcin, either excreted naturally or as a recombinant protein added to urine. The next objective of this study was to assess the importance of this male specific pheromone in the induction of female attraction and learned preferences for multiple male scent locations. This was achieved by addressing two questions:

a) Do competing male scents both need to contain darcin to stimulate a learned a preference in females?
b) When presented with multiple male scents that contain equivalent concentrations of darcin, do females respond differently according to whether the darcin is naturally produced or has been added to urine as a recombinant protein?

To answer the first of these questions, females were presented with male C57BL/6 urine and male BALB/c urine simultaneously. Male BALB/c urine contains negligible amounts of naturally produced darcin (Roberts *et al.,* 2010), and females do not demonstrate a learned preference for the location of this urine; by contrast, male C57BL/6 urine contains approximately 1μg/μl darcin and is known to induce a learned spatial preference for its location (Roberts *et al.,* 2012). Using a test comprising four locations, after confirming no location bias (no urine), female subjects were presented with the two urine stimuli (male BALB/c and male C57BL/6) and two control stimuli (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory).

During the learning session, there was a significant effect of stimulus on female attraction (repeated measures ANOVA: $F_{(2,20)} = 5.56$, $p = 0.012$). Females showed a significant attraction to the locations of both BALB/c and C57BL/6 male urine (BALB/c: $F_{(1,10)} = 6.22$, $p = 0.03$; C57BL/6: $F_{(1,10)} = 22.79$, $p = 0.001$; **Figure 3.7a**). Similarly, there was a significant effect of stimulus on female learned preferences ($F_{(2,20)} = 5.84$, $p = 0.01$), but in contrast to responses during the learning session, only male C57BL/6 urine induced a learned spatial preference for its location (C57BL/6 male

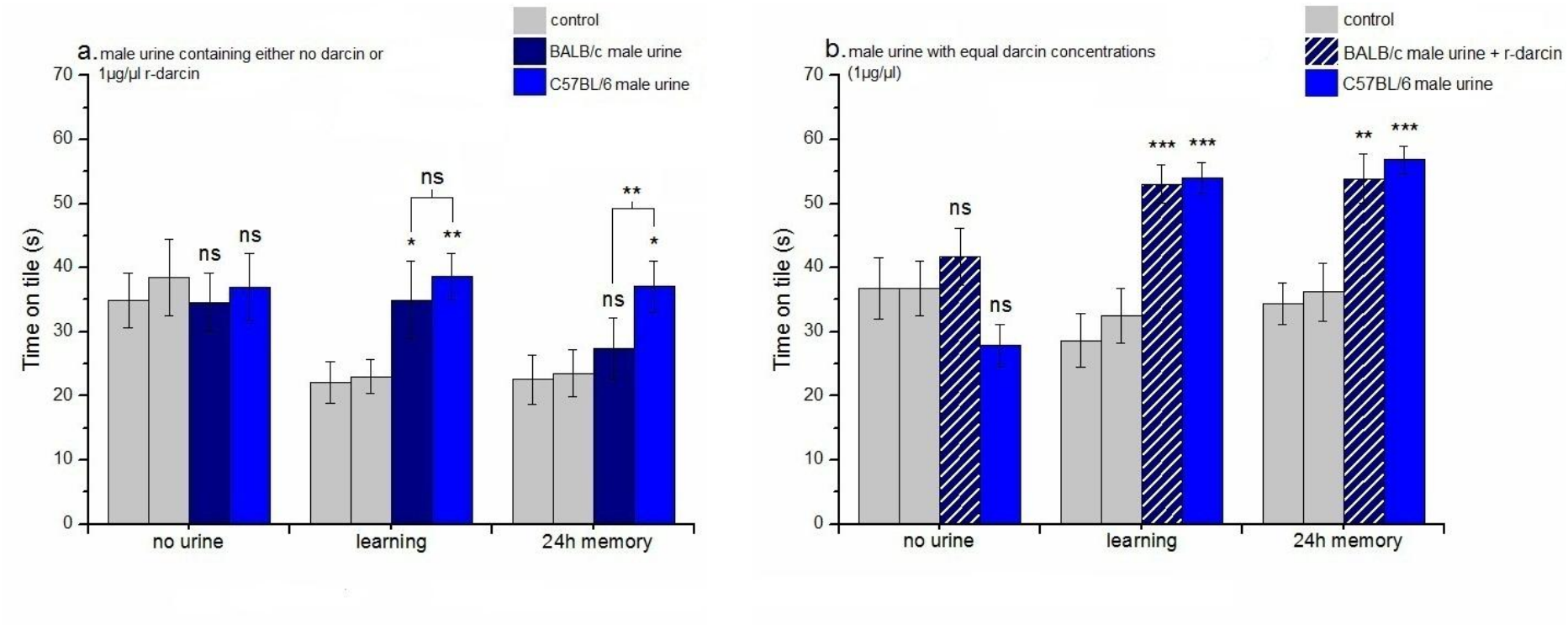

Figure 3.7 Female attraction and learned spatial preferences for multiple locations of male urine containing no darcin or 1µg/µl darcin.
Time spent by female subjects in urine or control locations when presented with (a) male C57BL/6 urine male and BALB/c urine or (b) male C57BL/6 urine and male BALB/c urine + 1µg/µl r-darcin. [(a) n = 11 subjects; (b) n = 12 subjects.] P values test for more time in urine versus mean time in control locations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

urine: $F_{(1,10)} = 7.76$, $p = 0.02$; BALB/c male urine: $F_{(1,10)} = 1.00$, $p = 0.34$; **Figure 3.7a**). Further contrasts revealed there was a significant difference in the remembered preference for C57BL/6 male urine compared to BALB/c male urine ($p = 0.008$). Although male urine from both laboratory strains induced a significant attraction during the learning session, only male urine containing darcin (C57BL/6) induced a learned preference for it spatial location when these scents were presented simultaneously.

To address the second question, a separate place preference test was conducted. When recombinant darcin is added to male BALB/c urine at a concentration of 1μg r-darcin per 1μl of urine, this scent conditions a learned preference for its location. However, it is unclear if male BALB/c urine containing 1μg/μl r-darcin conditions a learned preference for its location when presented simultaneously with male urine (C57BL/6) containing an equivalent amount of naturally expressed darcin. Further, whether the response to these two stimuli differs is also unclear. To address this using a test comprising four locations that allowed these scents to be presented simultaneously, after confirming no location bias (no urine), female subjects were presented with the two different urine stimuli (male BALB/c + 1μg/μl r-darcin and male C57BL/6) and two control stimuli (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory).

During the learning session, there was a significant effect of stimulus on female attraction (repeated measures ANOVA: $F_{(2,22)} = 38.71$, $p < 0.001$); females showed a significant attraction to both male urine stimuli (C57BL/6 male urine: $F_{(1,11)} = 40.68$, $p < 0.001$; BALB/c male urine + r-darcin: $F_{(1,11)} = 106.97$, $p < 0.001$; **Figure 3.7b**). There was no significant difference in attraction according to whether the urine contained recombinant or naturally produced darcin ($p = 0.75$). There was also an effect of stimulus on female learned preferences ($F_{(2,22)} = 18.67$, $p < 0.001$). Females demonstrated a learned spatial preference for both male urine stimuli (C57BL/6 male urine: $F_{(1,11)} = 35.44$, $p < 0.001$; BALB/c male urine + r-darcin: $F_{(1,11)} = 16.90$, $p = 0.002$; **Figure 3.7b**). There was no significant difference in the learned preference for male urine according to whether the urine contained recombinant or naturally produced darcin ($p = 0.38$). The male specific pheromone, darcin, appears to be important for the induction of female learned preferences for multiple locations of competing male scents. In addition, the responses of females to male urine containing equivalent levels of either recombinant darcin or naturally produced darcin did not differ.

3.3.5 Are female learned preferences for male urine with added recombinant darcin specifically due to the addition of darcin?

Addition of r-darcin to male BALB/c urine increases overall protein concentration of male urine as well as increasing darcin concentration specifically. Learned spatial preferences of females for male BALB/c urine containing r-darcin may therefore be influenced by this increase in protein concentration. To investigate this, a conditioned place preference test in which females were presented with multiple male BALB/c urine stimuli, each with the addition of a different r-MUP was conducted. After confirming no location bias (no urine), female subjects were presented with male BALB/c urine with the addition of r-darcin, male BALB/c urine with the addition of another male specific MUP, (r-MUP7), male BALB/c urine with the addition of a non-sex specific MUP (r-MUP11) and a single control stimulus (male BALB/c urine) during a single, 10-min learning session. All r-MUPs were added to urine at a concentration of 1µg per 1µl urine. Conditioned place preference was tested 24 hours later with no urine present (24h memory). By presenting females with multiple scent stimuli containing the same protein concentration, the response to darcin specifically could be assessed.

During the learning session, there was an effect of stimulus on female attraction (repeated measures ANOVA: $F_{(3,45)} = 6.73$, $p = 0.001$); females showed a significant attraction to male BALB/c urine containing r-darcin or r-MUP7, the male-specific MUPs (r-MUP7: $F_{(1,15)} = 7.10$, $p = 0.02$; r-MUP11: $F_{(1,15)} = 3.65$, $p = 0.08$; r-darcin: $F_{(1,15)} = 36.31$, $p < 0.001$; **Figure 3.8a**). When compared directly, attraction to urine containing r-darcin was significantly different from attraction to urine containing r-MUP7 ($p = 0.004$), but only showed a tendency to be different from attraction to r-MUP11 ($p = 0.11$). There was only a tendency for stimulus to influence learned preferences of females ($F_{(3,45)} = 2.03$, $p = 0.10$); only male BALB/c urine containing r-darcin conditioned a learned preference for its location (r-MUP7: $F_{(1,15)} = 0.82$, $p = 0.38$; r-MUP11: $F_{(1,15)} = 0.004$, $p = 0.95$; r-darcin: $F_{(1,15)} = 4.38$, $p = 0.04$; **Figure 3.8a**). In contrast to responses during the learning session, the learned preference for the location of urine containing r-darcin was not significantly different from the preference for urine containing r-MUP7 ($p = 0.38$), but was significantly different from the preference for urine containing r-MUP11 ($p = 0.006$). Females showed attraction to BALB/c male urine containing 1µg/µl of male-specific r-MUPs (darcin and r-MUP7), but a learned spatial preference for only male urine containing r-darcin.

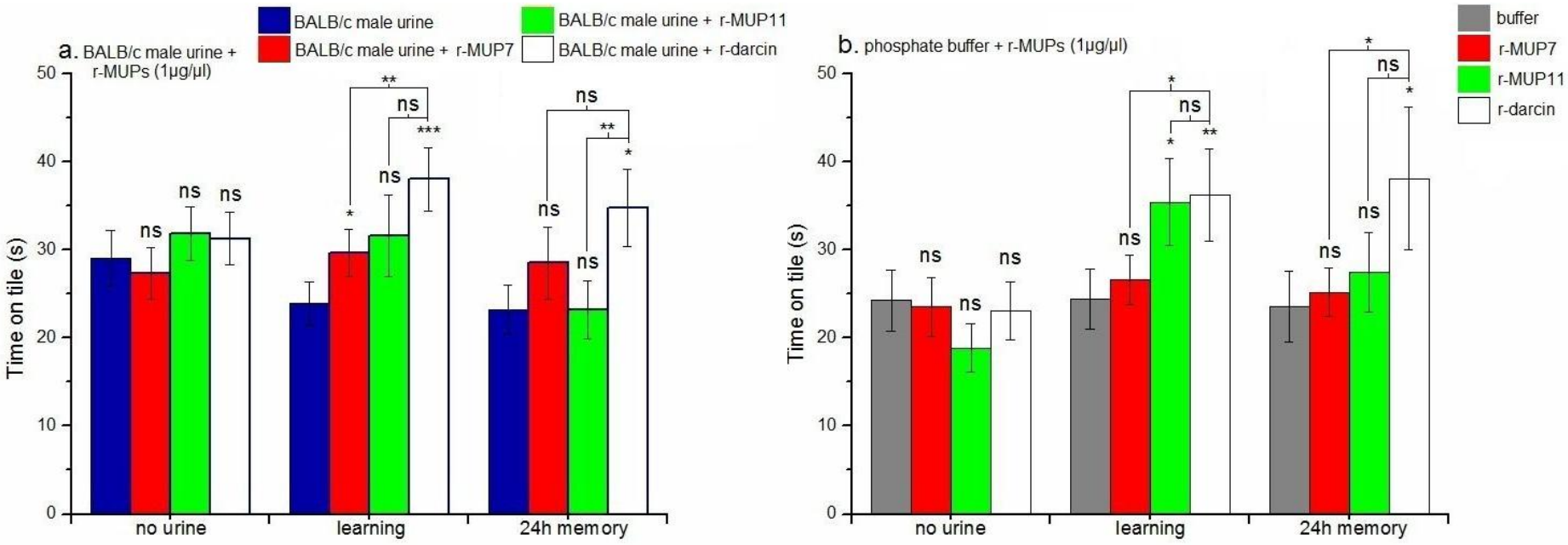

Figure 3.8 Female attraction and learned spatial preferences for different recombinant MUPs when added to male BALB/c urine or presented alone.

Time spent by female subjects in recombinant MUP or control locations when presented with (a) male BALB/c urine + r-MUPs or (b) r-MUPs. [(a) n = 16 subjects; (b) n = 11 subjects.] P values test for more time in r-MUP versus control location where control was (a) male BALB/c urine or (b) buffer. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3.6 Can darcin stimulate a learned preference for its location?

The male specific MUP, darcin stimulates a learned preference in wild female mice for its location (Roberts *et al.,* 2012). To establish whether this involatile pheromone can condition a learned spatial preference in female laboratory mice, a conditioned place preference test comprising four locations was used. After confirming no location bias (no urine), female subjects were presented with r-darcin, r-MUP7, r-MUP11 and a single control stimulus (phosphate buffer) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no r-MUPs present (24h memory).

There was an overall effect of stimulus on female attraction (repeated measures ANOVA: $F_{(3,30)} = 4.34$, $p = 0.012$), but females only showed a significant attraction to the location of r-darcin, and, in contrast to the response to recombinant MUPs added to male BALB/c urine, r-MUP11 (r-MUP7: $F_{(1,10)} = 0.52$, $p = 0.49$; r-MUP11: $F_{(1,10)} = 11.08$, $p = 0.008$; r-darcin: $F_{(1,10)} = 9.06$, $p = 0.01$; **Figure 3.8b**). When compared directly, the responses to r-darcin was significantly different from the response to r-MUP7 ($p = 0.03$), but was not different from the response to r-MUP11 ($p = 0.87$). However, despite there being a significant effect of stimulus on females learned preferences ($F_{(3,30)} = 3.90$, $p = 0.02$), only r-darcin conditioned a learned preference for its location (r-MUP7: $F_{(1,10)} = 0.01$, $p = 0.92$; r-MUP11: $F_{(1,10)} = 0.84$, $p = 0.38$; r-darcin: $F_{(1,10)} = 5.36$, $p = 0.04$; **Figure 3.8b**). Similarly to responses during the learning session, the learned preference for the location of r-darcin was significantly different from the preference for the location of r-MUP7 ($p = 0.03$), but was not significantly different from the preference for r-MUP11 ($p = 0.12$). Recombinant MUPs r-MUP11 and r-darcin induced a significant attraction in females, but only r-darcin induced a learned spatial preference for its location.

To assess whether the darcin protein is as potent a stimulator of learned spatial preferences in females as male urine containing this protein, the responses of females presented with r-MUPs or r-MUPs added to male BALB/c urine were compared. During the learning session, the significant attraction of females to the location of scent containing r-darcin did not differ significantly according to whether r-darcin was added to male BALB/c urine or presented without any other urinary components (independent Student's t-test: $t_{25} = -0.52$, $p = 0.61$). Similarly, the learned spatial

preference for the location of the stimulus containing r-darcin did not differ significantly according to whether r-darcin was added to male BALB/c urine or presented without any other urinary components ($t_{25} = 0.70$, $p = 0.49$). This suggests the darcin protein is as potent a stimulator of learned spatial preferences as male urine containing this protein even in this competitive context.

3.3.7 What is the minimum concentration of darcin required for male scent to condition a preference for its location?

Results presented in previous sections confirmed the specific importance of darcin for female learned preferences for male scent locations, even when females encounter multiple male scents. This suggests that investment in darcin within scent marks is an important aspect of male scent signalling. However, it is unknown what minimum concentration of darcin is required for females to form a learned preference for a male scent location, and how this compares to normal levels of male investment in this MUP. Results of previous tests showed that female mice demonstrate a learned spatial preference for the location of male C57BL/6 urine but not male BALB/c urine. C57BL/6 males naturally produce darcin at a concentration of approximately 1μg/μl; BALB/c males produce negligible levels (less than 0.5%) of this male specific pheromone (approximately 0.025μg/μl darcin) (Armstrong *et al.,* 2005; Roberts *et al.,* 2010). However, when r-darcin is added to male BALB/c urine at a concentration of 1μg/μl, females show a learned preference for the location of this urine. As a learned spatial preference for the location of male urine is not observed when darcin levels are negligible, the threshold concentration is likely to be between levels naturally expressed by C57BL/6 males and levels naturally expressed by BALB/c males.

To investigate what concentration of darcin must be present in male urine for it to induce a learned preference for its location, two separate groups of females were exposed to male BALB/c urine containing a range of r-darcin concentrations. Adding r-darcin to male BALB/c urine ensured this was the only differing variable between stimuli. After confirming no location bias (no urine), female subjects were presented with male BALB/c urine with the addition of different concentrations of r-darcin and a single control stimulus during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory).

When presented with male BALB/c urine containing 1μg/μl, 0.25μg/μl or 0.1μg/μl r-darcin simultaneously (i.e. 100%, 25% and 10% respectively of a concentration known to reliably stimulate a learned spatial preference), there was an overall effect of stimulus on female attraction (Friedman test: $\chi^2(3) = 17.20$, $p < 0.001$). Females showed a significant attraction to all male urine stimuli (Wilcoxon signed ranks test: 0.1μg/μl r-darcin: $Z = -2.98$, $p < 0.001$; 0.25μg/μl r-darcin: $Z = -3.06$, $p < 0.001$; 1μg/μl r-darcin: $Z = -2.82$, $p < 0.001$; **Figure 3.9a**); further, there was no significant difference in attraction according to darcin concentration ($\chi^2(2) = 0.17$, $p = 0.98$). There was only a tendency for stimulus to effect female learned preferences ($\chi^2(3) = 5.80$, $p = 0.12$), but females demonstrated a learned spatial preference for all male urine stimuli (0.1μg/μl r-darcin: $Z = -2.75$, $p = 0.002$; 0.25μg/μl r-darcin: $Z = -1.88$, $p = 0.03$; 1μg/μl r-darcin: $Z = -2.67$, $p = 0.002$; **Figure 3.9a**). Similarly to responses during the learning session, there was no significant difference in learned preferences for male urine according to darcin concentration ($\chi^2(2) = 0.17$, $p = 0.98$). Male urine containing r-darcin at a concentration of 0.1μg/μl induced a significant attraction and learned spatial preference for its location, even in the presence of other male scents containing higher concentrations of recombinant darcin. This suggests the threshold may be at a lower concentration than this.

To determine if male urine containing a lower concentration of darcin than 0.1μg/μl is able to condition a learned preference for its location, females were exposed to male BALB/c urine containing 1μg/μl, 0.05μg/μl or 0.025μg/μl r-darcin (100%, 5% and 2.5% respectively of a concentration known to reliably stimulate a learned spatial preference). During the learning session, there was a significant effect of stimulus on female attraction (repeated measures ANOVA: $F_{(3,33)} = 3.21$, $p = 0.036$), but females only showed a significant attraction to male BALB/c urine containing 1μg/μl r-darcin (0.025μg/μl r-darcin: $F_{(1,11)} = 0.43$, $p = 0.53$; 0.05μg/μl r-darcin: $F_{(1,11)} = 1.28$, $p = 0.28$; 1μg/μl r-darcin: $F_{(1,11)} = 5.73$, $p = 0.04$; **Figure 3.9b**). Similarly, there was a significant effect of stimulus on female learned preferences (repeated measures ANOVA: $F_{(3,33)} = 2.88$, $p = 0.05$), and only male BALB/c urine containing 1μg/μl r-darcin induced a learned spatial preference for its location (0.025μg/μl r-darcin: $F_{(1,11)} = 1.39$, $p = 0.26$; 0.05μg/μl r-darcin: $F_{(1,11)} = 0.23$, $p = 0.64$; 1μg/μl r-darcin: $F_{(1,11)} = 9.56$, $p = 0.01$; **Figure 3.9b**). Male urine with the addition of 0.1μg/μl r-darcin conditioned a learned preference for its spatial location; urine with the addition of 0.05μg/μl r-darcin did not.

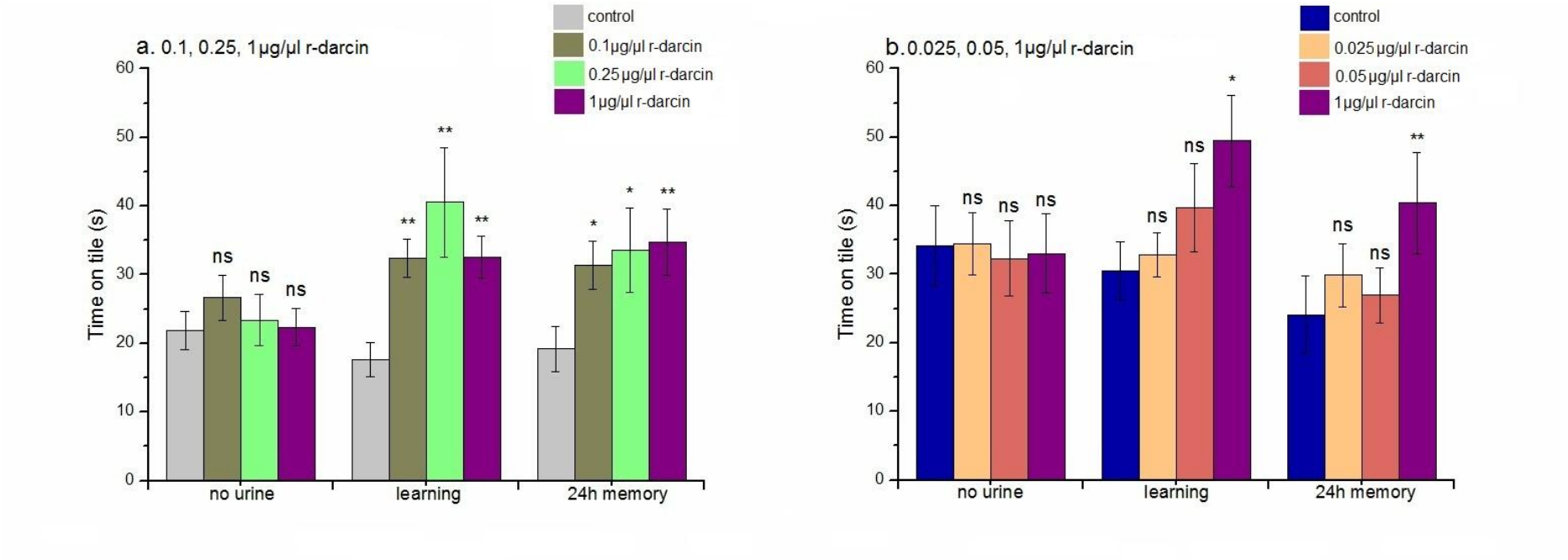

Figure 3.9 Female attraction and learned spatial preferences for male BALB/c urine containing different concentrations of r-darcin. Time spent by female subjects in urine or control locations when presented with (a) male BALB/c urine + 0.1µg/µl, 0.25µg/µl or 1µg/µl r-darcin or (b) male BALB/c urine + 0.025µg/µl, 0.05µg/µl and 1µg/µl r-darcin. [(a) and (b) n = 12 subjects.] P values test for more time in male urine versus control location where control was (a) ddH_2O or (b) male BALB/c urine. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM. ** $p < 0.01$, *** $p < 0.001$.

When presented with multiple male scents, females may assess and compare scents and only form a learned spatial preference for the most attractive scent or the one that is preferred above the others. To ensure that the lack of a learned spatial preference for the location of male urine containing 0.05μg/μl r-darcin was not influenced by the presence of male urine containing r-darcin at a concentration 20x greater (1μg/μl) in the same test, a conditioned place preference test comprising two locations was conducted. After confirming no location bias (no urine), female subjects were presented with male BALB/c urine with the addition of 0.05μg/μl r-darcin and a single control stimulus (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). During the learning session females did not show a significant attraction to the male urine (matched pair t-test: $t_{11} = 0.56$, $p = 0.59$). Similarly, male BALB/c urine containing 0.05μg/μl r-darcin did not induce a learned preference for its location ($t_{11} = 0.52$, $p = 0.62$; **Figure 3.10**). The minimum concentration of darcin required to induce a learned spatial preference in females for the location of male scent is therefore between 0.05μg/μl and 0.1μg/μl.

3.3.8 How does the estimated threshold concentration compare to normal darcin expression among wild male mice?

To determine how the estimated threshold concentration described above compares to the natural expression levels of wild male mice, the darcin concentration of urine collected from 40 wild stock males bred in the laboratory was measured. The protein concentration of urine samples was first assessed using the Coomassie plus protein assay kit from Perbio Science UK Ltd (Northumberland, UK). Samples were then diluted to 0.25μg/μl protein with ddH_2O and analysed using SDS-PAGE to separate out the darcin protein from the rest of the major MUPs present in urine. Densitometry was then performed on all bands visualised with Coomassie Brilliant Blue stain to estimate the percentage and concentration of darcin present.

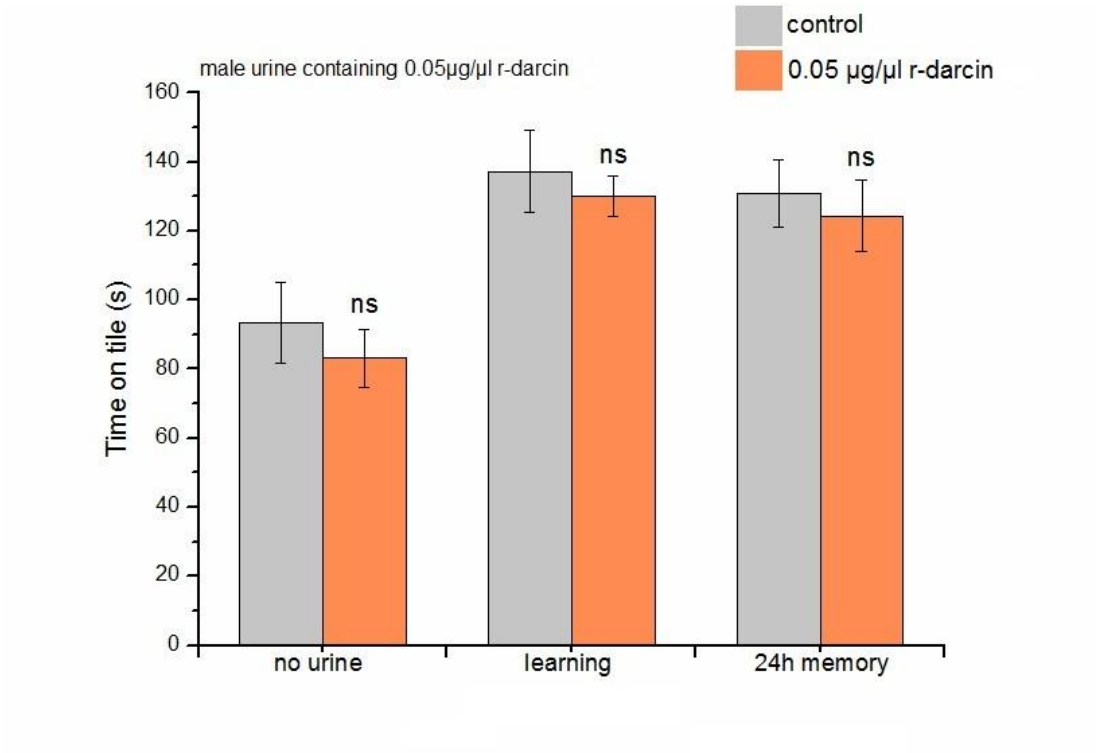

Figure 3.10 Female attraction and learned spatial preference for male BALB/c urine containing 0.05µg/µl r-darcin.

Time spent by female subjects in urine or control locations when presented with male BALB/c urine + 0.05µg/µl r-darcin [n = 12 subjects.] P values test for more time in male urine versus control location * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM. ** $p < 0.01$, *** $p < 0.001$.

The protein concentration of urine samples varied from 5.54μg/μg creatinine to 37.7μg/μg creatinine (4.01μg/μl to 21.7μg/μl when not adjusted for urinary dilution). Urinary darcin concentration varied from 0.59μg/μl to 4.78μg/μl (mean ± SEM = 1.82 ± 0.19μg/μl; **Figure 3.11a**); six male expressed concentrations approximately half or less than the mean and three males expressed concentrations approximately twice the mean, suggesting variation in darcin expression among males is high. The amount of darcin was positively correlated with total urinary protein (Spearman's rho: $r_s = 0.72$, $p < 0.001$; **Figure 3.11b**), but was not correlated with the percentage of overall protein expressed as darcin ($r_s = -0.10$, $p = 0.54$); further, darcin concentration was not correlated with urinary creatinine, a measure of urinary dilution ($r_s = 0.03$, $p = 0.88$). This suggests differences in darcin expression may have been due to individual differences in overall protein expression rather than differences between males in the proportion of overall MUP expressed as darcin. In addition, darcin was not linked to urine dilution; males excreting the lowest concentrations of darcin did not necessarily excrete the most dilute urine. As the wild stock males used in this study were housed individually, the pressures of competition with other males were absent. As a result, it is possible that the protein and darcin content of urine samples described here are much lower than could be expected under natural conditions, particularly as males increase their investment in MUPs under competitive conditions (Garratt *et al.,* 2012).

3.3.9 Do differences in darcin concentration between male scents influence female learned preferences for competing male scents?

Adult male mice consistently express high levels of darcin in their urine and under competitive conditions males increase investment in MUPs (Garratt *et al.,* 2012). The amount of darcin in a male scent may therefore be important for female assessment. Additionally, the VNO is likely to be important in detection of this protein due to its large, involatile nature, and stimulus strength may be encoded by corresponding activation of the VNO (Liman, 1996). As VNO outputs project to several brain regions, including those associated with olfactory and spatial learning, the level of stimulation of neurons in the VNO may influence upstream processing.

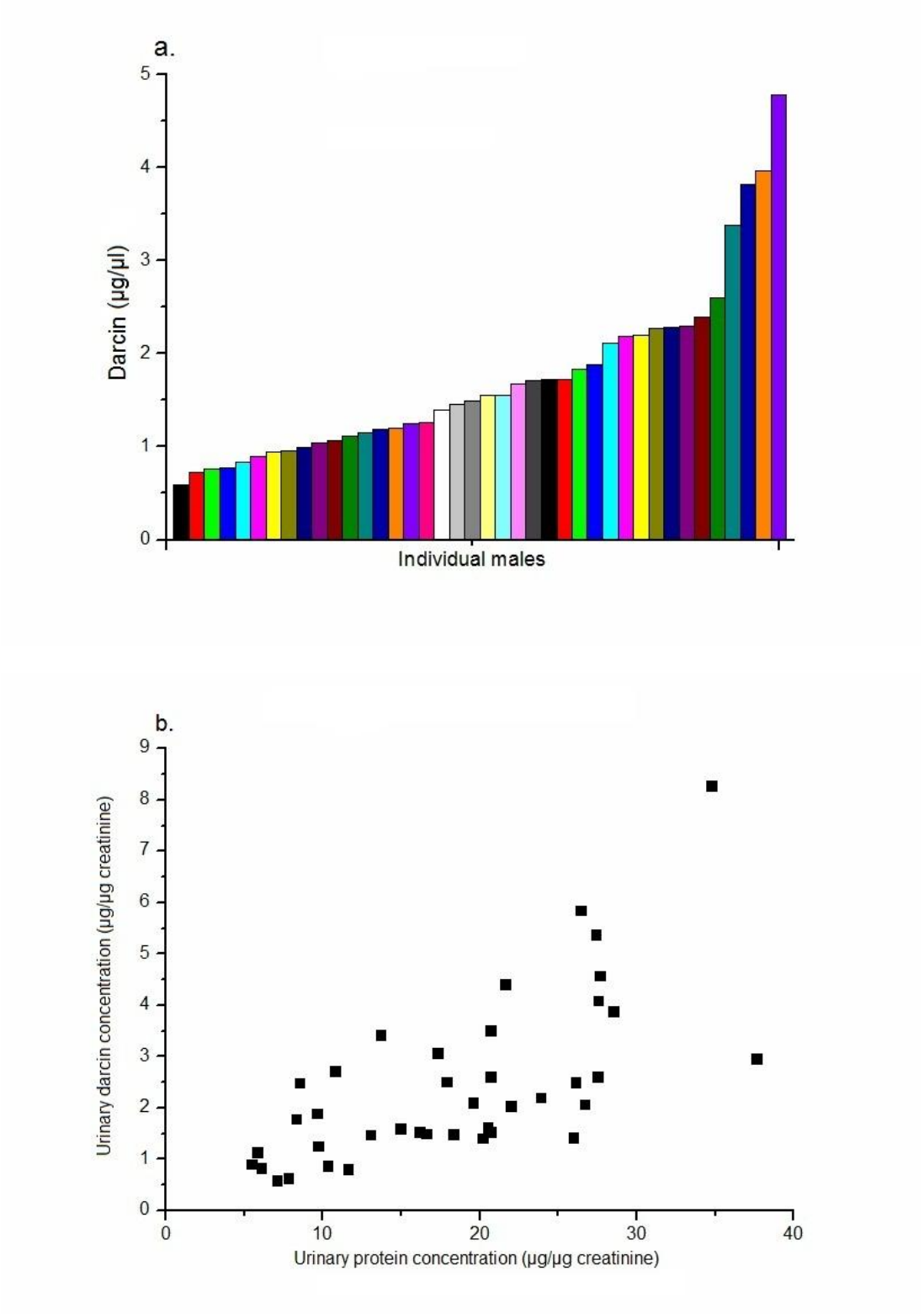

Figure 3.11 Darcin and protein concentrations of male urine

Urinary (a) darcin concentration (µg/µl) of samples collected from 40 individual wild males and (b) the positive correlation between darcin concentration and protein concentration in these samples ($r_s = 0.72$, $p < 0.001$).

The next objective was to assess how differences in darcin concentration of multiple male scents influences learned preferences of females for the locations of those scents. Using two separate conditioned place preference tests comprising four locations, after confirming no location bias (no urine), female subjects were presented with male BALB/c urine with the addition of different concentrations of r-darcin and a control stimulus (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). In each test, females were presented with male urine containing a 'low' concentration of darcin and male urine containing a 'high' concentration of darcin; in one test these scents differed in darcin concentration by 1μg/μl, and in a separate test these scents differed in darcin concentration by 2μg/μl. Analysis of female responses therefore allowed assessment of whether females respond differently to scents that contain different concentrations of darcin when multiple scents are compared, and if the magnitude of the difference in darcin concentration between the scents is important.

During the learning session, there was an overall effect of stimulus on female attraction (repeated measures ANOVA: $F_{(3,78)} = 10.70$, $p < 0.001$; **Figure 3.12**). Contrasts revealed there was no significant difference in female attraction to male urine containing high versus low concentrations of darcin ($F_{(1,26)} = 0.43$, $p = 0.52$); this was not influenced by the size of the difference in darcin concentration (interaction between stimulus and difference in darcin concentration $F_{(1,26)} = 0.76$, $p = 0.39$). Similarly, there was an overall effect of stimulus on female learned preferences ($F_{(3,78)} = 18.69$, $p < 0.001$). In contrast to responses during the learning session, when data from both tests were combined to provide an overall analysis of female responses, there was a significant difference in the learned preferences of females for male urine containing a high concentration of darcin versus male urine containing a low concentration of darcin ($F_{(1,26)} = 5.60$, $p = 0.026$; **Figure 3.12**). However, the size of the difference in darcin concentration between the scents did not influence this response (interaction between stimulus and difference in darcin concentration: $F_{(1,26)} = 1.14$, $p = 0.30$). Females showed a significant attraction to all male scents containing r-darcin, regardless of whether the scent contained a high or low concentration of darcin. By contrast, learned spatial preferences of females differed significantly according to whether the male urine containing a high or a low concentration of darcin, but this was not influenced by the magnitude of the difference in darcin concentration.

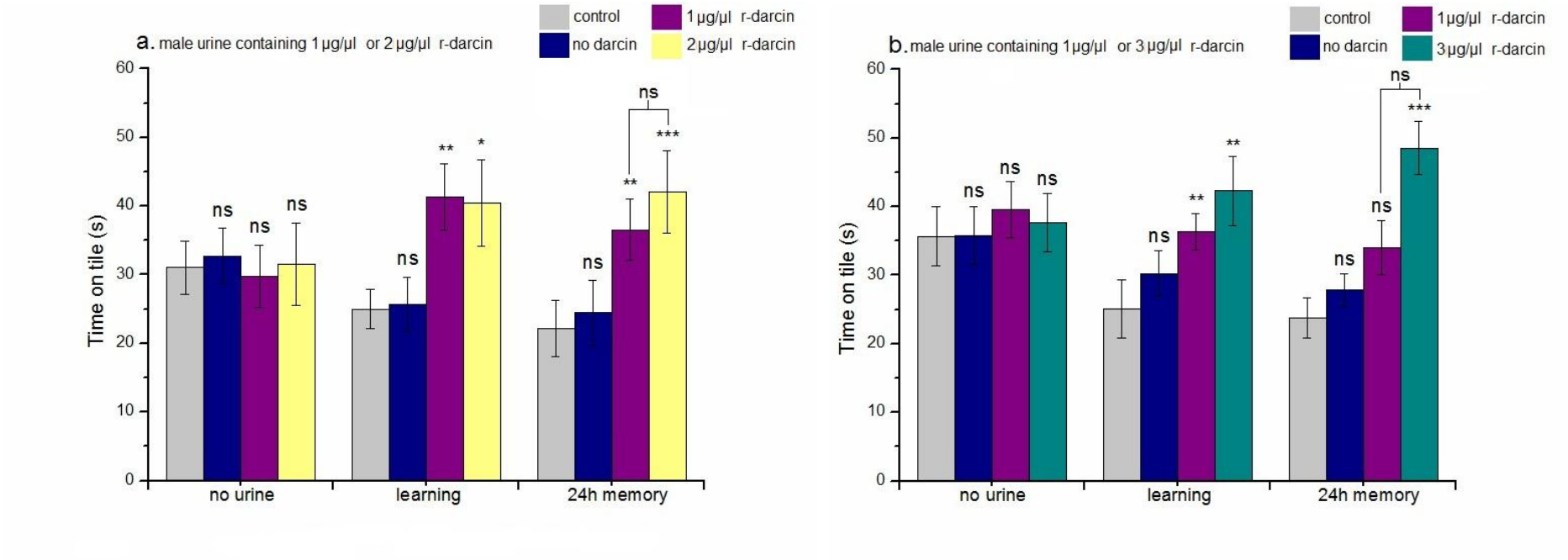

Figure 3.12 Female attraction and learned spatial preferences for male BALB/c urine containing different concentrations of r-darcin.
Time spent by female subjects in urine or control locations when presented with (a) male BALB/c urine + 0, 1µg/µl and 2µg/µl r-darcin or (b) male BALB/c urine + 0, 1µg/µl or 3µg/µl r-darcin. [(a) n = 11 subjects; (b) n = 17 subjects.] P values test for more time in male urine versus control locations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3.10 Does the age of a male scent influence female learned preferences?

Male urine containing darcin reliably stimulates a learned preference for its location. This male-specific MUP is responsible for binding the majority of the male-specific volatile compound 2-sec-butyl 4, 5 dihydrothiazole (Armstrong *et al.,* 2005), allowing extended release of this pheromone from male scent over many hours (Robertson *et al.,* 2001). Females continue to show attraction to a male scent that has been aged for up to 7 days (Roberts *et al.,* 2010), even though 98% of thiazole may be lost during the first 24h after a scent is deposited (Armstrong *et al.,* 2005). The age of a male scent therefore influences its composition, providing a potential mechanism by which females are able to distinguish between two scent marks based on scent age; typically females prefer the scent deposited most recently (Rich and Hurst, 1999). This may influence whether male scent induces a learned preference for its location.

To assess whether the age of a scent influences learned spatial preferences in laboratory mice, females were presented with either fresh male C57BL/6 urine, or male C57BL/6 urine that had been aged for 7 days. Using tests comprising two locations, after confirming no side bias (no urine), females were presented with a single stimulus urine (fresh or aged) and a control stimulus (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). Females showed a significant attraction to the location of C57BL/6 male urine when the urine was fresh (Wilcoxon signed ranks test: $Z = -1.88$, $p = 0.03$; **Figure 3.13a**), or had been aged for 7 days ($Z = 1.73$, $p = 0.05$; **Figure 3.13b**). Further, there was no significant difference in the attraction of females to the location of male urine according to the age of the scent (Mann-Whitney U test: $U = 63.00$, $Z = -0.52$, $p = 0.63$). Females also demonstrated a learned spatial preference for the location of C57BL/6 male urine that was fresh (Wilcoxon signed ranks test: $Z = 2.49$, $p = 0.005$; **Figure 3.13a**), or had been aged for 7 days ($Z = 2.35$, $p = 0.008$; **Figure 3.13b**); again there was no significant difference in learned preferences for the location of male urine according to the age of the scent (Mann-Whitney U test: $U = 65.00$, $Z = -0.06$, $p = 0.98$). When encountered individually, male urine conditions a learned preference for its location even when it has been aged for 7 days.

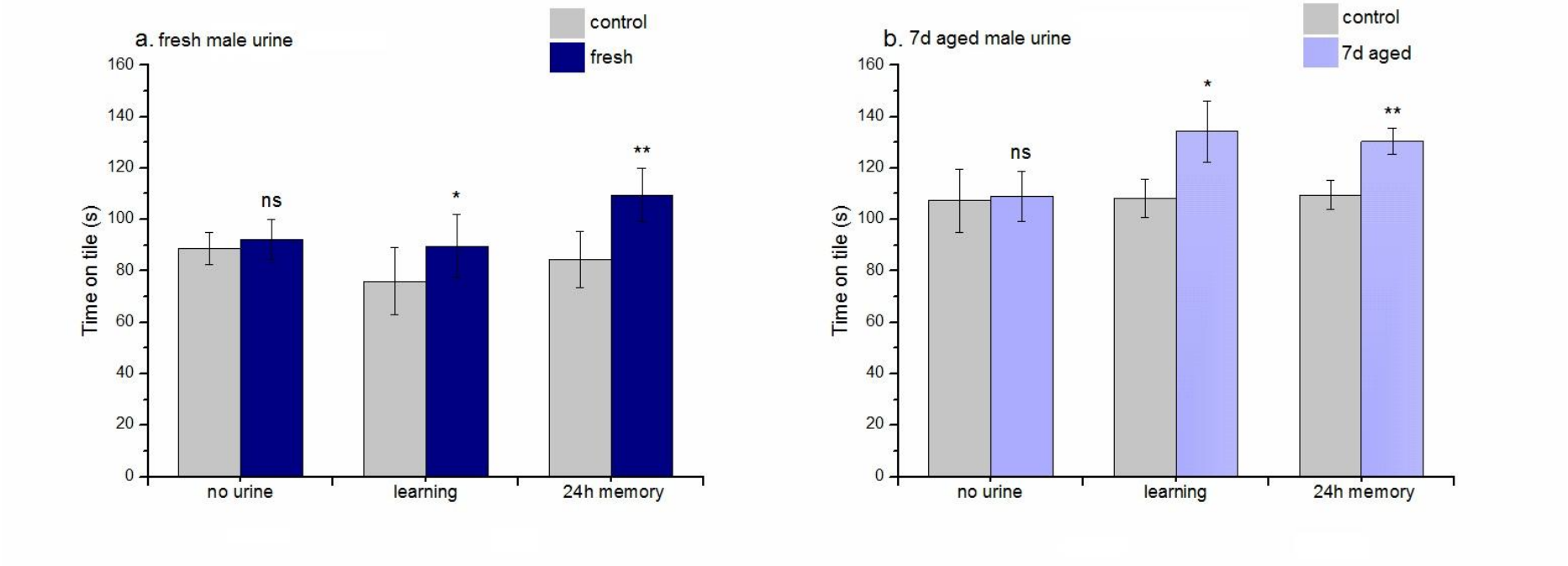

Figure 3.13 Female attraction and learned spatial preferences for fresh or aged C57BL/6 male urine.
Time spent by female subjects in urine or control locations when presented with (a) fresh male C57BL/6 urine or (b) male C57BL/6 urine aged for 7d. [(a) and (b) n = 12 subjects.] P values test for more time in male urine versus control locations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3.11 Does scent age influence female learned preferences for competing male scents that differ in age?

Male mice invest in competitive countermarking when intruders deposit scent marks within their territory and constantly refresh these marks to reinforce their dominance (Hurst, 1993). As a result, scent freshness indicates the ability of a male to effectively defend and maintain his territory. A remembered preference for the location of the freshest of multiple scents may increase the likelihood that a female will return to the territory of a male of high competitive ability and quality. As a result, despite the fact that females showed a learned preference for male scent that had been aged when this urine stimulus is presented alone, scent age may be more important for learned preferences in a competitive context. The role of scent ageing in a competitive context was addressed using a conditioned place preference test comprising four locations; fresh male C57BL/6 urine and male C57BL/6 urine that had been aged for 7 days was presented to females in the same test. After confirming no side bias (no urine), female subjects were presented with the stimulus urine (fresh and aged urine) and two control stimuli (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory).

During the learning session, there was a significant effect of stimulus on female attraction (repeated measures ANOVA: $F_{(2,22)} = 4.82$, $p = 0.02$). Females showed a significant attraction to fresh male C57BL/6 urine (fresh urine $F_{(1,11)} = 38.97$, $p < 0.001$; **Figure 3.14**), but this was not significantly different from the response to aged male urine ($F_{(1,11)} = 1.52$, $p = 0.24$). Similarly, there was a significant effect of stimulus on female learned preferences (repeated measures ANOVA: $F_{(2,22)} = 4.85$, $p = 0.02$). Females showed a learned preference for fresh male urine (fresh urine $F_{(1,11)} = 8.89$, $p = 0.01$; **Figure 3.14**), but there was only a tendency for this response to be different from to the response to aged male urine ($F_{(1,11)} = 2.66$, $p = 0.13$). Even when scents of differing age were presented simultaneously, attraction and learned spatial preference for male scent did not differ significantly according to the age of the scent.

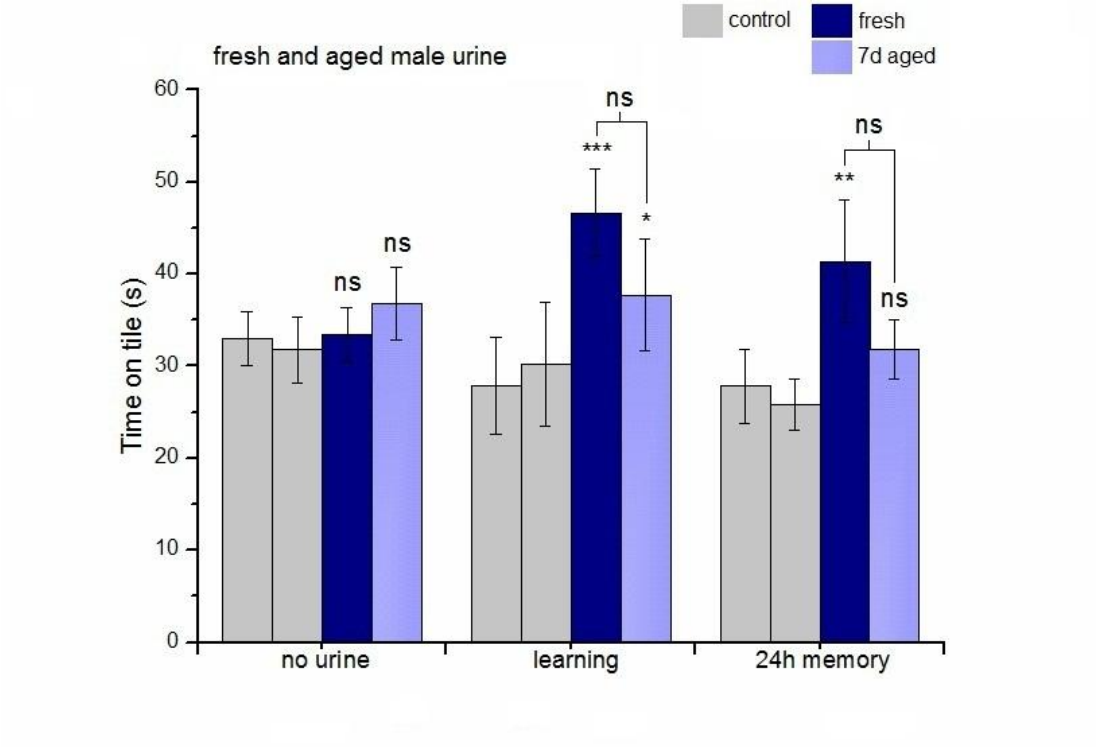

Figure 3.14 Female attraction and learned spatial preferences for fresh and aged C57BL/6 male urine presented simultaneously.

Time spent by female subjects in urine or control locations when presented with fresh and 7d aged male C57BL/6 urine (n = 12 subjects). P values test for more time in male urine versus control locations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

3.3.12 Does familiarity influence learned spatial preferences for male scent locations?

Females find the owner of familiar scents more attractive than scents of novel individuals (Cheetham, 2006). This preference may be beneficial as assessment of long term male quality in terms of health status and competitive ability may be more accurate if a male or his scent has been encountered several times. Males also defend scent-marked territories and exclude other males by countermarking intruder scents, informing females honestly of competitive ability; the continued presence of a single male's scent marks therefore indicates the high quality of a male (Hurst and Rich, 1999). As a result, females are more likely to come into contact repeatedly with the scent of a dominant local territory owner and familiarity may act as a proxy for quality (Cheetham *et al.,* 2008). Returning to the site of a familiar scent may assist females in selecting a mate of high quality. To assess the importance of prior familiarity on learned spatial preferences for male scent locations, urine collected from individual wild stock males was used in a conditioned place preference test comprising four locations. Unlike laboratory mice, which exhibit uniform expression of MUPs across individuals of each strain (Cheetham *et al.,* 2009), individual wild mice each express a unique MUP profile, providing a stable and reliable signature of individual identity in addition to other differences between the scents of individuals (Hurst *et al.,* 2001).

After confirming no side bias (no urine), prior to the learning session, females were exposed to urine from one individual wild stock male for 30 minutes; this urine was then considered familiar to subjects. Female subjects were then presented with the stimulus urine (familiar urine and a second, unfamiliar urine stimulus) and two control stimuli (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). During the learning session, there was a significant effect of stimulus on female attraction (repeated measures ANOVA: $F_{(2,22)} = 4.26$, $p = 0.03$; **Figure 3.15**). Females showed a significant attraction to the familiar male urine (familiar: $F_{(1,11)} = 9.15$, $p = 0.012$); this attraction was not significantly different from the attraction to the unfamiliar male urine (familiar versus unfamiliar: $F_{(1,11)} = 0.11$, $p = 0.75$). Similarly, there was a significant effect of stimulus on female learned preferences ($F_{(2,22)} = 7.70$, $p = 0.003$), and females showed a learned spatial preference for the familiar male urine (familiar: $F_{(1,11)} = 7.43$, $p = 0.02$). However, in contrast to the response during the learning session, there was a significant

difference in female learned preferences for the familiar versus unfamiliar male urine stimuli (familiar versus unfamiliar: $F_{(1,11)} = 12.37$, $p = 0.005$). Whilst females demonstrated a similar attraction to both familiar and unfamiliar male urine, only familiar male urine conditioned a learned spatial preference for its location.

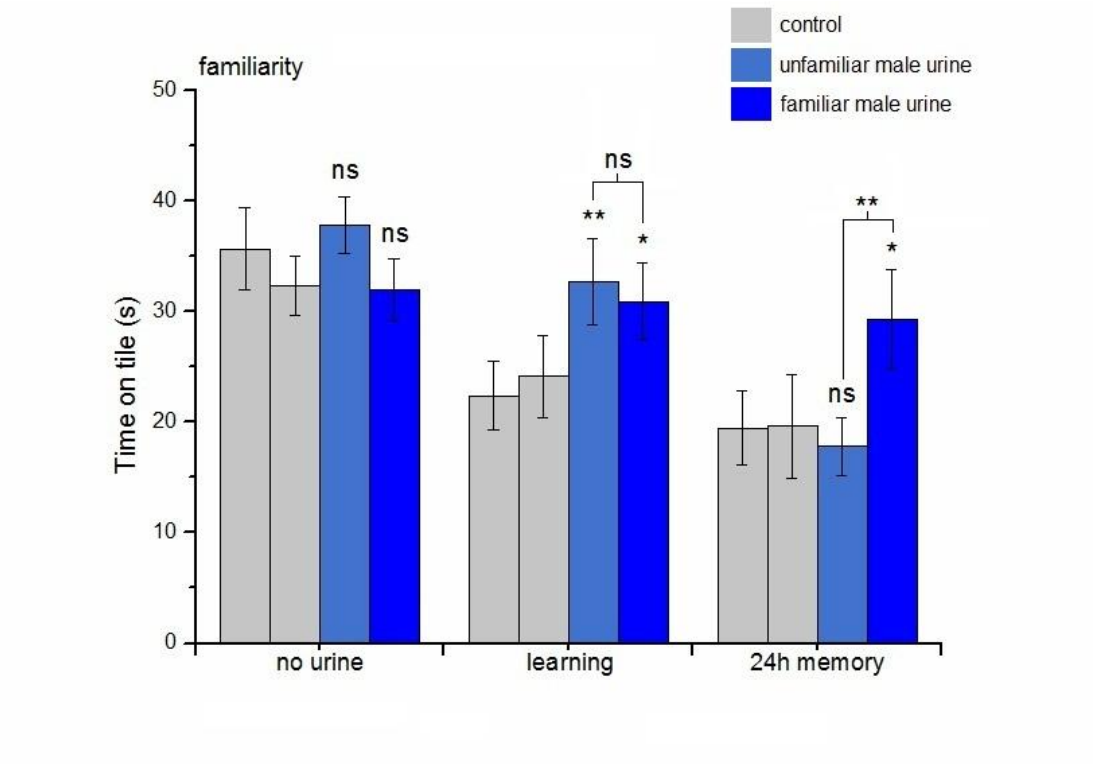

Figure 3.15 Female attraction and learned spatial preferences for familiar and unfamiliar wild male urine.

Time spent by female subjects in urine or control locations when presented with familiar and unfamiliar male urine ($n = 12$ subjects). P values test for more time in male urine versus control locations * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM

3.4 DISCUSSION

The overall aims of this study were: (1) to investigate learned spatial preferences of female laboratory mice for conspecific scents, and to confirm that the same scents and scent components that stimulate learned spatial preferences in wild mice also stimulate this response in laboratory females; (2) to assess how the comparative composition of individual scents influences female learned preferences for locations scent marked by competing males. These aims were achieved by presenting a single scent to female subjects in conditioned place preference tests comprising two locations, or by using conditioned place preference tests comprising four locations to allow multiple scents to be simultaneously presented to females in a competitive context.

3.4.1 Learned spatial preferences of female laboratory mice for single scents.

The first objectives of this study were to assess whether the scent that stimulates learned spatial preferences in laboratory mice for conspecific odours is in male urine, and to confirm that the male-specific MUP, darcin, is important for this response in laboratory females. When presented to females as a single scent stimulus during a single learning session, male but not female urine conditioned a preference for its remembered location. Further, when females were presented with male urine containing negligible levels of darcin, conditioning of a place preference did not occur. However, addition of recombinant darcin to this urine restored a conditioned spatial preference. The results of these tests indicate darcin is important for male odour-conditioned spatial preferences in female laboratory mice. This is consistent with previous findings by Roberts *et al.*, (2012) showing that wild female mice only show a learned preference for male urine and that darcin is required for male scent to condition a preference for its location. However, compared to results presented by Roberts *et al.* (2012), the strength of responses of female laboratory mice to male urine was lower. For example, when conditioned place preference was assessed, wild female mice spent approximately three times longer in the location where laboratory male urine (C57BL/6) was previously encountered compared to the control location (Roberts *et al.*, 2012); data presented here show laboratory female mice spent approximately 1.3 times longer in the location of male urine compared to the control stimulus. This suggests wild female mice respond much more strongly to male scents and may form stronger learned preferences for their

location. However, this difference may be due to a number of factors, including differences in the behaviour of wild and laboratory female mice during tests, and the method used to measure the amount of time females spent in the scent locations. Female laboratory mice typically exhibit a greater level of exploratory behaviour within the confines of a novel test arena than wild mice and are less likely to exhibit behaviour such as thigmotaxis (the tendency to remain close a vertical surface). As a result, laboratory mice are likely to spend much greater periods of time on the tiles in the test arena as they move around and investigate the arena, regardless of the location of the urine stimulus. By contrast, wild female mice may only move into the central area of an arena and thus the locations around the scent stimuli to directly investigate the scent. In addition, Roberts *et al.* (2012) recorded the time females spent in the petri dishes containing stimuli; female laboratory mice used in the tests in the study presented here were typically too large to fit comfortably inside the petri dishes and the majority of females failed to spend any time sitting in any of the dishes. As time spent on the tile surrounding the stimulus was quantified in the study outlined here and the tiles cover a much greater proportion of the test arena than the petri dishes, time spent on the tiles would be high, even when behaviour is not directed at the urine stimulus. As a result, females spend relatively long periods of time even on the tile surrounding the control stimulus.

3.4.2 Female learned spatial preferences for competing male scents

To assess remembered spatial preferences after females encounter multiple scents in different locations, conditioned place preference tests comprising four stimuli in four locations were used. When multiple scents were presented simultaneously, females formed a learned preference for multiple locations of male but not female scents. The results also show that darcin, a male-specific pheromone, was required for male scent to condition a preference for its remembered location when females were presented with multiple scents. Further, the response to male urine containing equal amounts of naturally produced or recombinant darcin did not differ.

When presented alone (i.e. not in the context of other urinary components) darcin stimulates a learned preference for its location in wild derived mice (Roberts *et al.,* 2012). Similarly, in the experiments presented here, when three recombinant MUPs

were presented to females, only r-darcin conditioned a learned preference for its location. However, although r-MUP11 did not condition a preference for its location, unexpectedly, females showed a significant attraction to this scent during the learning session. It is possible that the presence of this non-sex specific and therefore familiar MUP encouraged females to investigate. However, novel scents typically stimulate increased levels of investigation, so r-MUP7 might also be expected to stimulate such a response, although this was not observed. One female spent an unusual 71s in the location of r-MUP11, approximately double the mean time other subjects spent in the location of this stimulus. An unusual response of females to this recombinant MUP was observed in a previous study; when assessing the attraction of females to recombinant MUPs, Roberts *et al.,* (2010) found the response of females to r-MUP11 showed abnormally high variability due to one of the subjects showing an unusually high attraction to this MUP. Although the unexpected response to r-MUP11 was observed when r-MUPs were presented without being added to male urine, in the test where this MUP was added to male BALB/c urine no attraction or learned preference was observed. It is unclear why females spent so much time investigating this scent, and it is possible that this outcome may simply have occurred as a result of chance, particularly as this increased attraction during the learning session did not lead to a conditioned preference for the scent location. Despite this unexpected result, these tests show when multiple scents are presented in a competitive context, darcin is both necessary and sufficient to stimulate a learned preference for a scent location regardless of prior attraction.

Female mice in a natural context would explore the territories of multiple males, often encountering many different male scents in close proximity to each other. The importance of darcin for learned preferences would be particularly useful; this MUP is male-specific and naturally produced by all sexually mature wild males, so would allow females to immediately target the most informative scents for recognition and assessment of potential mates. Darcin also provides a mechanism for learning the associated volatile olfactory signature of a scent (Roberts *et al.,* 2010), meaning females may only need to remember the approximate location of a preferred scent; the recognised volatile signature could then guide females to the exact location. This would save time and energy in the search for potential mates.

3.4.3 Urinary darcin concentration and female learned preferences for competing male scents

Male scent containing 1μg/μl r-darcin reliably conditions a preference for its location. However, male BALB/c urine, which contains negligible levels of naturally produced darcin, does not. To identify the minimum concentration of darcin that must be present for male urine to condition a preference for its location, females were presented with male urine containing darcin concentrations as low as 0.025μg/μl. The results show male scent containing 0.1μg/μl, but not 0.05μg/μl, is able to condition a preference for its location. The threshold level of darcin is therefore likely to be between these values. V2 receptors in the VNO have been shown to respond to the high molecular weight fraction of urine containing MUPs such as darcin (Chamero *et al.,* 2007), and VNO receptors that respond to other urinary components such as farnesene and thiazole are highly sensitive. The detection threshold for these pheromones is remarkably low (approximately 10^{-11} M) meaning these VNO neurons are among the most sensitive chemodetectors found in mammals (Leinders-Zufall *et al.,* 2000). Although a single receptor that responds to darcin has not yet been identified, as receptors respond to single molecules, detection of darcin and the bound volatile compound thiazole could occur even when very low concentrations are present in urine. However, it is possible that stimulation of a certain proportion of receptors must first occur for higher order olfactory and spatial processing to occur, leading to stimulation of a learned spatial preference for a scent. Additionally, the presence of a simple detection threshold may be beneficial to females. The production of male pheromones is often under the control of a range of hormones including testosterone; the presence of a threshold level may ensure females do not respond inappropriately to juveniles, subordinate individuals or aged males that are no longer able to express high levels of this important pheromone.

Analysis of urine samples collected from 40 wild male mice bred and housed in the laboratory revealed that darcin expression ranged from approximately 0.5 – 4.7μg/μl (1.81μg/μl ± 0.19; mean ± SE mean) and the mean concentration of overall protein in the urine of these males was 18.4mg/mg creatinine. However, all males used as urine donors were housed individually, and thus the pressures of competition and establishing a social hierarchy were removed. This suggests that in a natural context, expression of MUPs and darcin may reach levels even higher than those found here. In

support of this, one study showed that wild males under competitive conditions express MUPs at levels two to three times greater than isolated males; further, wild males under competitive conditions have been shown to express protein at a concentration as high as approximately 30mg/mg creatinine (Garratt *et al.,* 2012). This suggests expression of protein is increased under competitive conditions. As analysis of urine collected from the 40 males used in this study showed darcin concentration was positively correlated with urinary protein concentration, males may also increase darcin expression under competitive conditions when overall protein is increased. As a result, the amount of this specific MUP in urine may be important for how males assess one another and for female assessment of male scents. When females were exposed to male scents containing high or low concentrations of darcin simultaneously, the strength of the remembered preference for the locations of the scents differed; females showed a stronger learned preference for the scent containing a high concentration of darcin. This was not influenced by the magnitude of the difference between the scents (i.e. the relative amount of darcin). This suggests that when females encounter competing male scents that differ in darcin concentration, females may assess the concentration of darcin present and show a stronger learned preference for the location where the scent containing the most darcin was deposited. This lends support to the theory that this is a competitive signal, and may be sexually selected and an important signal in mate choice.

Protein production is likely to be energetically expensive for males (Gosling *et al.,* 2000), imposing the highest costs on males that produce the greatest quantities. The amount of darcin present in a scent may therefore be an important indicator of male quality; darcin may act as a proxy signal for the protein content of a scent, allowing females to potentially choose males based on their ability to withstand the costs of high levels of protein expression. MUP production is also under the control of a range of hormones including testosterone (Knopf *et al.,* 1983). As a result, darcin concentration may additionally reflect subtle underlying differences between individuals in testosterone levels; high testosterone levels are often associated with dominance, aggression and the ability of a male to defend his territory. However, the links between darcin expression and social status are unclear. In addition, higher levels of darcin may result in the presence of larger quantities of thiazole, the volatile pheromone bound by this protein. Thiazole is an androgen-dependent volatile component of scent marks that attracts females to sniff male scents and can induce oestrus (Jemiolo *et al.*, 1985). The

binding of this component by darcin slows its release, extending the life of the scent mark (Hurst *et al.*, 1998). A high concentration of darcin would not necessarily extend the life of a scent mark further, as each protein molecule has the same capacity to bind thiazole; rather, the presence of more darcin may result in a greater concentration of thiazole being released, thus increasing the airborne volatiles in the vicinity of a scent. As a result, females may be able to detect scents more readily or from a greater distance.

The amount of pheromone a male produces is important in several species, particularly in insects. For example, female arctiid moths (*Utetheisa ornatrix*) prefer to mate with males that produce the greatest amount of a pheromone derived from alkaloids that males consume in their diet (Dossourd *et al.,* 1991). Alkaloids are of high nutritional benefit and during mating males transfer a portion of their alkaloid reserves to females. A male with higher levels of this pheromone transfers more alkaloids for the provision of offspring and thus increase a female's reproductive success. Similarly, female tobacco moths (*Ephestia elutella*) prefer to mate with males that produce high levels of wing-gland pheromone. These matings typically result in a greater number of offspring with increased survival rates (Phelan and Baker, 1986).

Detection of involatile pheromones such as darcin occurs in the VNO. Each receptor in the VNO detects a limited number of pheromonal components (Leinders-Zufall *et al.,* 2000), allowing behavioural responses to these chemical signals to be finely tuned. Exposure of VNO neurons to different concentrations of male mouse pheromones *in vitro* results in a concentration-dependent electrical response (Leinders-Zufall *et al.,* 2000). However, the spatial activation of the VNO organ is not influenced by pheromone concentration. This suggests as pheromone concentration increases, the number of responding neurons does not change, but the electrical response of each individual neuron increases. Although a specific receptor for darcin within the VNO has not yet been identified, a mixture containing recombinant MUPS that included r-darcin has been shown to stimulate V2 receptors in the VNO (Chamero *et al.,* 2007). It is therefore possible that increasing concentrations of a large volatile protein such as darcin may result in increased stimulation of the neurons in the VNO and AOB. In turn, this may stimulate increased activity in the regions of the brain that process olfactory information, including the hippocampus. As a result, a stronger association between a scent and its location may be formed, enhancing the learned spatial preference for a scent containing a high concentration of darcin.

3.4.5 Learned spatial preferences for scents that differ in age

To investigate how scent ageing influences conditioning of learned preferences for male scent locations, tests were conducted with male scents aged for up to 7 days. When presented as a single urine stimulus, male urine conditioned a preference for its location, regardless of whether it was fresh or had been aged for 7 days. Similarly, when these scents were presented simultaneously, there was no significant difference in the response to fresh and aged male urine. These results suggest females did not respond preferentially to fresh scent when comparing competing scents of differing age. Male territory owners expend time and energy adding many small marks in the local area around any intruder scents, often returning repeatedly to re-mark over the course of several hours (Humphries *et al.,* 1999). This maximises the rate of replenishment, which in turn ensures the scent of the dominant territory holder remains the freshest in any given location. Only males able to tolerate the high metabolic costs of constantly replenishing scent marks could ensure their scent remains the freshest in a given location. It could therefore be beneficial to females to return to only the locations where they have encountered the freshest scents, enhancing the chances of encountering a dominant male territory owner or a male of high quality and competitive ability. The result obtained in these tests was therefore unexpected. Despite this, it is possible that the urine stimuli used in this test may have contributed to the unexpected result. Urine was collected from male C57BL/6 mice, and, as a result of generations of inbreeding, individuals within this laboratory strain display identical MUP patterns in their urine (Cheetham *et al.,* 2009). It is possible that females perceived both scent stimuli, regardless of age, as being deposited by the same individual, thus representing marks of different age at distinct locations within the overall territory of that male. To fully understand the influence of scent age on female learned preference for competing male scents, it would be more appropriate in future tests to present females with fresh and aged urine collected from different individual wild males.

However, the fact that females did not respond differently to fresh and aged scents indicates the binding properties of darcin may be important for female attraction and learning of locations of aged scents. Darcin is responsible for binding the majority of the male-specific volatile 2-sec-butyl 4, 5 dihydrothiazole (Armstrong *et al.,* 2005). The barrel-like structure of darcin tightly binds volatile thiazole at its centre, allowing for an extended release of this pheromone from male scent over many hours

(Robertson *et al.,* 2001). Consistent with the results presented here, females continue to show attraction to a male scent aged for up to 7 days (Roberts *et al.,* 2010), despite the fact that after 24h approximately 98% of the thiazole may be lost (Armstrong *et al.,* 2005). The extended release of this volatile pheromone is therefore a key function of darcin, allowing male scent to remain active as an attractant long after the scent has been deposited. Alternatively, darcin itself or other unidentified volatile components may be important for female attraction and learning of aged scents.

3.4.6 Scent familiarity and learned spatial preferences

When presented with familiar and novel urine simultaneously, females only demonstrated a learned spatial preference for the location of familiar male urine; remembering the location of a familiar scent may allow females to subsequently locate a dominant local territory owner when ready to mate. Male countermarking of intruder scents excludes other males from a territory and informs females honestly of the competitive ability of the resource holder (Rich and Hurst, 1999). The continued presence of a single male's scent marks therefore indicates the high quality of that male. Females are therefore more likely to come into contact repeatedly with the scent of a dominant local territory owner and familiarity may act as a proxy for quality (Cheetham *et al.,* 2008). In mice, females find the owner of a familiar scent more attractive than a novel individual (Cheetham, 2006), and there are a range of potential benefits that may arise from a preference for the scent of a familiar individual. For example, accurate assessment of long term quality in terms of health status and competitive ability may be more successful if a scent signal is encountered and assessed on multiple occasions.

The response of females to familiar but not novel male scent raises an issue that is applicable to all tests carried out in this study. In all tests, the amount of time females spent in the immediate vicinity of a scent was recorded. This method appears reliable even in the complex 4-location tests. However, this measure does not discriminate a failure in memory formation from females simply choosing not to spend more time in the location where a scent has been encountered. For example, it is clear that male BALB/c urine does not condition a preference for its location in any of the tests conducted with this scent as a stimulus. This scent may not induce a memory for its location and as a result, females do not show a learned preference for the location.

Alternatively, this scent may induce a memory for its location, but it is not preferred or attractive, so subjects do not show a learned preference for its location when this is subsequently assessed. A lack of time spent investigating scents that do not contain darcin or are presented simultaneously with other preferred scents may mediate the lack of a learned preference. However, when multiple male scents were presented together, the mean amount of time females spent sniffing and closely investigating male urine containing darcin was very similar to that spent investigating male urine containing negligible levels of darcin (i.e. C57BL/6 and BALB/c), at 7.2s and 7.7s respectively. Similarly, although only familiar male urine induced a learned preference for its location when presented simultaneously with novel male urine, the time females spent sniffing these stimuli during the learning session was similar, at 3.7s and 3.1s respectively. These data suggest that the amount of close contact sniffing and investigation of a scent may not be an important mediator of a subsequent learned preference in laboratory mice.

Results presented in this chapter show darcin is necessary for male scent to induce learning and spatial memory, but that other factors influence the behavioural response in concert with delivery of this protein to the accessory olfactory system. Subsequent higher order processing in regions such as the piriform cortex and hippocampus is likely to be important for memories of scent locations, particularly as the hippocampus plays an important role in spatial memory. The hippocampus contains 'place cells', so called due to the stimulation and excitation of these cells that is dependent upon an animal's location within the environment (O'Keefe and Dostrovsky, 1971). The neurons within the hippocampus itself are activated by inputs from most sensory modalities including the olfactory system. As a result, the hippocampus does process sensory information, but only the spatial attributes of this information. Activation of specific neurons within a given location may therefore be mediated by scent components such as darcin.

The hippocampus is also one of the few regions of the brain where neurogenesis, or the generation of new neurons, persists throughout life. Neurogenesis in this region is important for spatial memory and learning in a range of tasks (Shors *et al.,* 2002; Leuner *et al.,* 2006) and may be important in mate selection. Odour from dominant males stimulates neurogenesis in the hippocampus and olfactory bulb, whilst odour from castrated or subordinate males does not (Mak *et al.*, 2007). This response is also time dependent; two days of exposure does not stimulate an increase in

neurogenesis but seven days of odour exposure does. Despite this requirement for prolonged exposure to stimulate neurogenesis, male scent can condition a preference for its location with exposures as brief as ten minutes. This suggests neurogenesis may not be involved in the process of forming spatial memories of scent locations after brief encounters with scent. Further, even though male scent stimulates both spatial learning and adult neurogenesis in females, it is unclear if the same scent components are important for both of these processes. Establishing the role of specific scent components in the stimulation of neurogenesis by male scent may provide important information regarding how olfactory and hippocampal processing combine to mediate olfactory and spatial memories that are important to females.

3.4.7 Conclusions

The presence of darcin in male scent underlies the ability of females to form remembered preferences for locations where male scents are encountered, representing an important mechanism by which spatial learning of rewarding natural stimuli can occur in a natural context. The darcin pheromone alone was able to condition a preference for its location, suggesting it may be a particularly significant social signal in the attraction and learning of female mice in response to the presence of male scent in specific locations. The ability of darcin to induce learning, even in a competitive context, adds a further function to the repertoire of this pheromone; it also mediates learning of the volatile odours associated with the specific scent of an individual male (Roberts *et al.,* 2010). This suggests a potentially important new role for pheromones in stimulating more than one type of associative learned response – to both the specific volatile profile of a scent and to the spatial location of a scent. This type of learning may be more common than currently known, particularly as the ability to quickly and easily form a learned preference for the location of scent marked sites would enhance the ability of females to locate potential mates. The fact that this response is not only rapid, but incredibly reliable, provides an important model that could be used to investigate changes in neurobiology as a result of exposure to important social odours, potentially providing evidence for how social odours are processed, stored, retrieved and responded to.

CHAPTER 4: Male scent signals that influence adult neurogenesis in female mice

4.1 INTRODUCTION

The integration of newly generated neurons persists throughout life in two structures of the adult mammalian brain: the olfactory bulb and the hippocampus. Neurogenesis is thought to be involved in the formation of olfactory and spatial memories and for most mammals, scents provide essential cues for recognition and assessment of conspecifics, particularly in the context of mate choice. As a result, the brain faces an important challenge. Olfactory signals may contain components that signal both fixed and variable information that the brain must process simultaneously (reviewed by Eisenberg and Kleiman, 1972). For example, the innate response to fixed species and sex information requires neurological circuitry to be maintained. Conversely, the response to factors that are variable such as competitive ability and health status must remain plastic, implying that underlying circuits must also be flexible and able to adapt. Additionally, the brain must constantly adjust to the growth of an individual and its environmental experiences during early development. Ultimately, the adult brain must maintain behaviour and preserve the underlying neural networks whilst allowing other important circuits to remain plastic and adaptable.

Scent plays an essential role in social communication in many species, including mice, and olfactory cues produced by males provide valuable information to females when assessing potential mates. In a natural context, when searching for mates, females may investigate the scents of multiple males (Hurst, 1987), assessing them and learning the olfactory identity signatures of numerous individuals. It would therefore be beneficial to remember individual male scent signatures and the locations of these scents, allowing females to be selective and approach preferred males when ready to mate. The neural systems involved in this olfactory discrimination and learning may therefore include the olfactory system and the hippocampus, the primary sites of adult neurogenesis. New olfactory neurons are generated in the subventricular zone of the forebrain and are thought to function in olfactory discrimination and memory. Additionally, hippocampal neurons generated in the dentate gyrus play a role in successful completion of learning and memory tasks, particularly when a spatial component is included. However, little is known about the role of neurogenesis in social recognition and assessment or if this process is influenced by the same scent components that mediate learning of individual scent signatures and their locations.

Exposure to male odours stimulates an increase in the generation of neurons in the olfactory bulb and hippocampus of females (Mak *et al.,* 2007; Nunez-Parra *et al.*, 2011). This suggests that female neurogenesis may play a role in processing information in male scent. Further, odours from dominant males stimulate neurogenesis but odours from castrated or subordinate males do not; blocking olfactory neurogenesis also removes a preference for a dominant male (Mak *et al.*, 2007) and impairs mate recognition (Oboti *et al.,* 2011). The generation of new neurons during adulthood in the olfactory system and hippocampus may therefore be influenced by scent components important for individual recognition and assessment. The overall aim of this study was to assess whether male scent components important for spatial and olfactory learning also stimulate hippocampal and olfactory neurogenesis in females.

4.1.1 Scent signals in mice

Male mice are often absent when females first explore their territories. However, males invest heavily in territorial scent marking; the location and pattern of male marks provides accurate details of the social status and competitive ability of an individual territory owner. Dominant males typically deposit a combination of large and small urine streaks at numerous sites around their territory (Desjardins *et al.,* 1973), often utilising specialised hairs on the prepuce to increase the area covered by each streak (Maruniak *et al.,* 1975). Dominant territory owners also ensure their own marks predominate (Hurst, 1993). These scent marks are attractive to females and signal sex and individual identity (Hurst *et al.*, 2001) as well as health and social status (Gosling and Roberts, 2001). Female mice strongly prefer to mate with dominant male territory owners having assessed status and identity through scent distribution and composition.

As discussed in more detail in section 1.3.2.1, scent components signal important information to females, and components such as the major urinary proteins (MUPs) provide a stable signal of genetic and individual identity (Rich and Hurst, 1999; Hurst *et al.,* 2001). MUPs are encoded by a highly polymorphic complex of genes and the pattern of these proteins excreted in the urine of mice is genetically fixed and stable throughout life (reviewed by Beynon and Hurst, 2003). Males invest more heavily in MUPs than females and testosterone plays a role in the hormonal control of differential expression of MUPs such that some are male specific (Knopf *et el.,* 1983; Armstrong *et*

al., 2005). These proteins therefore play an important role in individual recognition and assessment of genetic heterozygosity by females (Hurst *et al.,* 2001; Sherborne *et al.*, 2007).

MUPs have a central cavity that binds signalling pheromones such as thiazole and brevicomin (Novotny, 2003). Due to the tight binding of these volatile components, MUPs extend the life of a scent mark (Hurst *et al.*, 1998), allowing the scent ownership signal to persist in the environment long after the scent has been deposited. Darcin, a large, involatile MUP, is responsible for binding most of the male-specific volatile thiazole (Armstrong *et al.*, 2005), extending the release of this pheromone over several hours (Robertson *et al.,* 2001). Contact with darcin stimulates a learned attraction to the associated volatile components of the scent of an individual male, allowing inherent as well as selective attraction to individual scent marks (Roberts *et al.*, 2010). Adult males consistently express high levels of darcin in their urine and this pheromone plays an important role in female learning of male scent locations. Male scent can condition a learned preference for its remembered location but only if the scent contains darcin; further, this single MUP is as potent in stimulating a learned preference for a scent location as intact male urine (Roberts *et al.,* 2012). This spatial and olfactory memory may be beneficial to females, allowing them to be selective and return to the territory of a preferred male when ready to mate. Although darcin is known to be important in mediating this memory, little is known about the importance of this component for the neurological processes that may influence olfactory learning and spatial memory formation.

4.1.2 Combined olfactory and hippocampal processing

The processing of scent components, including darcin begins with the detection of odorant molecules by the main olfactory epithelium (MOE) and accessory vomeronasal organ (VNO). The signals produced during activation of neuron receptors within these regions are then processed by the main and accessory olfactory bulbs respectively. Subsequent higher-order processing occurs in the primary and accessory cortices including the entorhinal cortex (reviewed by Baum, 2009), which is a key source of input to the hippocampus (Tamamaki and Nojyo, 1995). The hippocampus is important in memory formation and spatial navigation and this link may be important

for olfactory learning and the storage of paired odour and spatial information (Witter and Amaral, 1991; Jarrard, 1995; Eichenbaum, 2000). Hippocampal function is therefore important for the completion of tasks involving spatial navigation, including those that involve olfactory-dependent learning (Li *et al.*, 1999; Gilbert and Kesner, 2002; Wood *et al.*, 2004). In addition, this region has been shown to be essential for social investigation and recognition in rodents (Maaswinkel *et al.,* 1996; Bannerman *et al.*, 2001; Uekita and Okanoya, 2011), behaviours typically mediated by olfactory signals. The main and accessory olfactory bulbs also project to regions that secrete vasopressin, a hormone that strongly regulates social memory in the hippocampus (reviewed by Bielsky and Young, 2004). This suggests the hippocampus is not only important for learning and spatial navigation, but that processing of some socially relevant odour signals and the spatial attributes of these signals may also rely on hippocampal function.

4.1.3 Adult Neurogenesis

For many social mammals, scent is essential for communication. The complexity of many olfactory signals requires that both fixed and variable signals be processed simultaneously. The brain must also adjust to the growth of an individual and its environmental and social experiences during early development. The adult brain therefore faces a significant challenge: maintain behaviour and preserve the underlying circuits whilst allowing other circuits to remain plastic and able to adapt. Neurogenesis, which can broadly be defined as the generation of new neurons, occurs in mammals throughout life and is suggested to play a role in maintaining the balance between stability and plasticity. Until the 1960s it was a widely held belief that neurogenesis only occurred in the brains of developing embryos and ceased following early post-natal development.

> "In the adult centres, the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree." – Santiago Ramon y Cajal (1913).

The discovery of neurogenesis in the brains of adults challenged the assumption that overall brain structure could not be altered once postnatal development was complete. In the 1960s, the first evidence of the occurrence of neurogenesis in adult rats was

published (Altman and Das, 1965; Altman 1969). Since then, neurogenesis has also been found to occur in many other species (reviewed by Taupin and Gage, 2002). The regions of the adult brain that support neurogenesis on a substantial level are the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles in the forebrain. The dentate gyrus controls input flow through the hippocampus (Yeckel and Berger, 1990), an area important for learning and spatial memory (Jarrard, 1995). By contrast, neural cells generated in the SVZ migrate to the olfactory bulb where they become incorporated into existing bulbar networks (Lois and Alvarez-Buylla, 1993).

4.1.3.1 Olfactory and hippocampal neurogenesis

In the brains of adult mammals, new neurons are added to the olfactory bulb throughout life and are generated within the SVZ, the cellular layer found within the walls of the lateral ventricles in the forebrain (Luskin, 1993; Lledo and Saghatelyan, 2005). The continual production of neurons throughout life requires that this region contain a large population of neural stem cells; approximately 30,000 new cells, or neuroblasts, are produced daily within the SVZ of mice (Lois and Alvarez-Buylla, 1994). Additionally, in mice, the SVZ is located several millimetres from the olfactory bulb meaning newly generated cells must migrate to the mature bulbar networks (Lois and Alvarez-Buylla, 1994). The process of migration is further complicated by the fact that migrating cells must traverse a complex network of interconnected pathways before joining the migration pathway known as the rostral migratory stream (RMS) (Doetsch and Alvarez-Buylla, 1996). Following birth, the cells organise into chains and join the RMS where blood vessels provide anchorage (Lois and Alvarez-Buylla, 1994; Whitman *et al,* 2009). Cells generated within the SVZ begin to differentiate prior to migration and continue this process as they move towards the olfactory bulb. When they reach the middle of their target, cells detach from their chains, migrate radially and begin terminal differentiation. The initial stages of proliferation and migration take approximately 7 days. Cessation of migration within the olfactory bulb usually occurs around day 9 when the final position of cells within the existing circuits is established. At this stage the cells have a simple structure, but over the next 2 to 20 days the morphology of cells changes; by day 30 the cells have a mature neuronal morphology (reviewed by Abrous *et al.*,

2005). Approximately 10,000 new neurons are added to the olfactory bulb on a daily basis (Winner *et al*., 2002) despite the death of approximately 50% of these cells during the first 45 days after generation. The surviving cells mature and integrate into existing circuits, staying functional for up to a year (Winner *et al.,* 2002).

Although a role for the olfactory bulb (OB) as a chemoattractant structure has been proposed, OB removal does not prevent neuronal precursors from migrating. However, in the long-term, without a target to move towards, neuroblasts accumulate in the RMS and cell death ensues (Kirschenbaum *et al*, 1999). Similarly, transection of the RMS or removal of the most rostral regions of the OB leads to continued migration but ultimately cells accumulate at the lesion site (Alonso *et al*, 1999). The presence of a working olfactory bulb is therefore not required for correct migration of neuroblasts, but olfactory activity does influence proliferation and survival. For example, olfactory enrichment results in an increase in proliferation rate within the SVZ (Alonso *et al*, 2008).

In contrast to olfactory neurogenesis, the germinal zone for hippocampal neurons is not located near the walls of the ventricles. Instead, the germinal subgranular zone (SGZ) is located in the dentate gyrus (DG), deep within the hippocampus. The process of neurogenesis within the DG comprises several developmental stages and at each stage distinct proteins are expressed and the developing cells exhibit specific morphologies and patterns of activity (Kempermann *et al*, 2004). The early stages involve stem cell division followed by multiple stages of proliferative activity. Over time, the proliferative activity of cells decreases in conjunction with an increase in differentiation towards a neuronal fate. The final stages comprise exit from the cell cycle and the creation of dendritic connections within the existing network (Zhao *et al.,* 2006). During the final postmitotic stages, differentiation of the newly generated cells into functioning neurons occurs. By 7 weeks post-generation, these cells are indistinguishable from the pre-existing neurons within the hippocampal circuitry (van Praag *et al*., 2002).

4.1.3.2 Regulation of adult neurogenesis

Studies of adult neurogenesis are broadly categorised into two distinct areas of interest; regulation of neurogenesis and the functional relevance of this process. In terms of neurogenesis regulation, mate choice and reproductive behaviour have a potent influence on cell proliferation and survival in females, mediated by hormones secreted as a result of exposure to potential mates or their scents. Exposure to male scent and pheromones induces oestrus (Whitten *et al.,* 1958), accelerates puberty (Novotny *et al.,* 1999) and promotes learning of individual scent signatures (Roberts *et al.,* 2010). As a result, increases in the rate of secretion of gonadal hormones may occur. For example, female oestrogen release is cyclic and increases dramatically during the proestrus phase of the oestrus cycle. Similarly, a surge of oestrogen and progesterone is typical at the onset of puberty. Other hormones, such as those produced by the adrenal gland may also be influenced by changes in reproductive physiology; the circadian rhythm of corticosterone release persists during the oestrus cycle, but typically increases during proestrus (Nichols and Chevins, 1981).

Oestrogens are produced primarily by the ovaries and by the Leydig cells in the testes and although these hormones are secreted in greater quantities in females, small quantities of circulating estradiol occurs in male rodents, as testosterone can be converted to this hormone via aromatase (Simpson *et al.,* 1994). These hormones influence the rate of both hippocampal cellular proliferation and survival (Tanapat *et al.,* 1999; Galea *et al.,* 2006), and changes in the rate of neurogenesis often occur synchronously with fluctuations in oestrogens over the course of the oestrus cycle in adult females. During the proestrus stage of the cycle when oestrogen is high, the number of proliferating cells in the hippocampus reaches the highest level. This is in contrast to the low levels of proliferation observed during diestrus (Tanapat *et al.*, 1999). This natural fluctuation is a major factor in the gender difference observed in most species of rodents; females typically have higher overall rates of neurogenesis than males (Tanapat *et al.*, 1999). However, results of studies exploring the influence of oestrogens on hippocampal neurogenesis in mice are inconsistent (Lagace *et al.,* 2007); cellular proliferation has even been reported to be lower in females during the breeding season when levels of oestrogen are at their highest (Ormerod and Galea, 2001). This suggests the influence of oestrogens on neurogenesis may involve a complex mechanism that includes other hormones such as serotonin and corticosterone

(Antonopoulos *et al.,* 1997; Ormerod *et al.,* 2003). Additionally, the limited availability of oestrogen receptors on newly generated cells in the hippocampus may modulate the influence of oestrogens on adult neurogenesis (Perez-Martin *et al.,* 2003).

Adrenal steroids such as corticosterone inhibit the production of new neurons by suppressing cellular proliferation (Desouza *et al.,* 2005). These hormones are primarily released into the bloodstream following the activation of the hypothalamo-pituitary-adrenal axis (HPA), a response associated with stress; the impact on neurogenesis is little influenced by the type of stress (e.g. isolation, restraint or exposure to predator odour) (reviewed by Mirescu and Gould, 2006). However, corticosterone levels also fluctuate following a circadian rhythm or in synchrony with changes in other hormones during the female oestrus cycle. In rodents the main steroid produced is corticosterone, which regulates its own secretion through negative feedback via receptors within the dentate gyrus of the hippocampus (Reul and Kloet, 1985). When corticosterone levels increase, a reduction in the number of proliferating cells occurs; conversely, suppression of corticosterone release increases neuron generation in the hippocampus (Cameron and Gould, 1994). This increase in proliferation occurs 24 hours after hormone secretion is suppressed and remains relatively constant for seven days. However, the overall rate of cell death is enhanced (Cameron and Gould, 1994), providing a feedback system designed to maintain a constant neural volume in the hippocampus. Short-term release of stress hormones is adaptive, reallocating energy to tasks that are essential for survival and restoration of homeostasis. However, prolonged high levels of these hormones may be detrimental to survival. Prolonged elevation of corticosterone has profound effects on many regions of the body, including the hippocampus, which is important for spatial cognition and long-term individual recognition memory (Kogan *et al*., 2000; Bannerman *et al*., 2012). Corticosterone-mediated changes in neurogenesis may therefore influence hippocampal function and the ability of individuals to respond appropriately to conspecific olfactory signals.

Although corticosterone and oestrogen levels fluctuate in a circadian rhythm or in response to conspecific signals, some of the most dramatic changes in these hormones are observed during pregnancy and parturition. In rodents there is an increase in estradiol during late pregnancy and a subsequent decrease during parturition (Rosenblatt *et al.,* 1988). Additionally, cellular proliferation is increased in the SVZ of the forebrain within the first few days of gestation (Shingo *et al.,* 2003), an effect

mediated by prolactin, another hormone that is elevated during pregnancy (reviewed by Larsen and Grattan, 2012). This hormone is important for stimulating milk production in the mammary glands, for the immune tolerance of mothers to the foetus (Grattan and Kokay, 2008), and is crucial for the expression of maternal behaviours (Bridges *et al.,* 1997). Reduced prolactin levels eliminate the typical 40% increase in forebrain neurogenesis that occurs during the first 7 days of pregnancy. Prolactin-mediated changes in olfactory neurogenesis as result of pregnancy may be therefore functionally relevant for the normal expression of maternal behaviour. The effect of pregnancy on hippocampal neurogenesis is less prominent, possibly due to a reduction in cellular proliferation and survival during the later stages of pregnancy and in the early postpartum period (reviewed by Pawluski *et al.,* 2009a). This may be mediated by a surge in corticosterone that occurs in late pregnancy (Pawluski *et al.,* 2009b), again reflecting the importance of interactions between hormones in the regulation of adult neurogenesis.

In addition to the effects of a number of hormones, the overall rate of neurogenesis declines with age. Ageing can have a significant impact, reducing the rate of hippocampal proliferation in the DG by up to 80% (Kuhn *et al.,* 1996). By contrast, the age-related decline in neurogenesis in the SVZ is less severe; SVZ neurogenesis decreases by around 50% compared to that seen in juveniles (Maslov *et al.,* 2004). The majority of age-related changes in the rate of hippocampal proliferation are observed between 3 and 12 months of age, but the percentage of cells surviving to maturity remains unaffected (Bondolfi *et al.,* 2004). Neuronal differentiation is also delayed as ageing advances. The overall mechanism that drives the age-related decline in hippocampal neurogenesis is unknown, but a decrease in responsiveness to environmental stimulation or an accumulation of inhibitory factors over time may occur. Administration of proteins that support neurogenesis such as growth factors can partially reduce the effects of ageing and increased environmental stimulation can delay the age-related decline in neurogenesis (Kuhn *et al.,* 1996; Luo *et al.,* 2006).

External environmental factors also influence the rate of adult neurogenesis. These include: learning, physical exercise and environmental enrichment (reviewed by Fuchs and Gould, 2000). Rodents living in an enriched environment within a laboratory typically have higher numbers of hippocampal neurons than individuals living in standard conditions (Kempermann *et al.,* 1997). This increased environmental

complexity is important in regulating cellular proliferation and survival, and similarly, learning influences neurogenesis. Training for tasks that require acquisition of spatial memory results in an increase in the number of new neurons in the DG (Gould *et al.,* 1999); the Morris water maze task is particularly effective in stimulating this change. This task involves training animals to use spatial cues to locate a hidden platform submerged beneath the surface in a pool of water (Morris, 1984). Rats able to use external cues to learn the location of the platform exhibit an increase in the number of new neurons within the DG of the hippocampus; training for as little as 4 days can stimulate a twofold increase in new neurons due to enhanced cellular survival (Drapeau *et al.,* 2003).

The Morris water maze is thought to be a particularly effective learning paradigm due to the additional effect of the increased physical activity in the form of swimming required to complete the task. Physical activity itself influences neurogenesis in rodents, and mice that regularly use running wheels exhibit a higher overall number of hippocampal neurons than more sedentary individuals (van Praag *et al.,* 1999). However, in contrast to changes in neurogenesis associated with learning, exercise-induced increases in neuron number are due to an increase in the rate of proliferation as well as survival (van Praag *et al.,* 1999). In some laboratory strains there is even a positive correlation between the distance run in wheels and overall rates of proliferation and cell survival (Clark *et al.,* 2011). However, this only occurs in strains that show dramatic individual variation in use of running wheels or in populations that have been bred to show extreme hyperactivity or sedentary behaviour. Learning also influences adult neurogenesis in the olfactory system in tasks that include an odour component (reviewed by Breton-Provencher and Saghatelyan, 2012). For example, cell survival rates increase in mice required to memorise unfamiliar odours (Breton-Provencher *et al.*, 2009), or that are trained to locate food rewards based on the presence of a specific odour in the reward location (Sultan *et al.,* 2010). Conversely, naris closure and olfactory deprivation results in a decrease in recruitment of new neurons to the bulbar network and in the survival rate of newly generated neurons in the SVZ (Corotto *et al.,* 1994).

Another component of the external environment that regulates neurogenesis is social interaction and experience. For most mammalian species, contact with conspecifics is important for group cohesion, parent-offspring relationships, competitive interactions and mate choice. For mice, much of the communication

between conspecifics is mediated by scent (reviewed by Arakawa *et al.,* 2008). Many aspects of social behaviour in rodents influence neurogenesis (reviewed by Gheusi *et al.,* 2009) and even the earliest forms of social experience can be important for this process. Young rodents reared in isolation typically show impairments in learning and memory that correlate with a reduction in the number of newly generated neurons within the hippocampus (Lu *et al.,* 2003). However, subsequent social interaction can rescue neurogenesis, although the mechanism underlying this process remains unclear (Lu *et al.,* 2003). Social stress may also be influential later in life through competition for resources, shelter and mates. The social defeat paradigm is commonly used to mimic social stress in adult males (reviewed by Martinez *et al.,* 1998); a resident male is provided with a territory to establish, occupy and subsequently defend rigorously against unfamiliar intruder males. To mimic subsequent intrusions, an unfamiliar male is placed into the cage of a dominant territory owner for a brief period, usually 1-2 minutes. The intruder is then removed and housed behind a mesh barrier, maintaining visual and olfactory contact with the resident territory owner. In this paradigm, intruder males show a robust stress response involving activation of the HPA axis and secretion of corticosterone. This type of chronic social stress subsequently depletes the rate of cellular proliferation in the DG (Mitra *et al.,* 2006). More specifically, the extent to which proliferation is reduced is strongly correlated with the frequency of defensive behaviour exhibited by the intruder male (Yap *et al.,* 2006).

Hippocampal and olfactory neurogenesis are also both influenced by sexual behaviour. Sexual experience enhances hippocampal neurogenesis in male rats (Leuner *et al*., 2010) and the associated odour signals that are rewarding for males (Tenk *et al.,* 2009) may mediate changes in cellular proliferation. Male odours also influence hippocampal neurogenesis in females. Exposure to the pheromones of dominant, but not subordinate or castrated males stimulates an increase in cellular proliferation within the hippocampus of female mice (Mak *et al*., 2007). These mice exhibit a prolactin mediated increase in neurogenesis within the SVZ and a subsequent enhancement of new neuron survival (Mak *et al*., 2007). This effect is time dependent; a 2-day exposure to male pheromones is insufficient to stimulate an increase in neurogenesis, but after 7 days of exposure the response is observed (Larsen *et al*., 2008). This response may be mediated by a number of factors including changes in hormone levels, which occur as a result of stimulation of oestrus or puberty by male scent. However, it is unclear whether

specific scent components or pheromones are responsible for this increase, or if an association between the hippocampal and olfactory neural networks is important for normal female responses to male scents.

4.1.3.3 The functional relevance of neurogenesis

Neurogenesis is regulated by a variety of factors, but investigating the functional relevance of new neurons is essential to understanding why this process persists in adults. Typically this is addressed via ablation of neurogenesis in the region of interest. Ablation of hippocampal neurogenesis prevents the acquisition of some, but not all learning tasks, suggesting that neurogenesis may be selectively involved in specific aspects of learning. For example, the amount of fear acquired after exposure to a trace fear conditioning paradigm is reduced by ablation of neurogenesis, but spatial navigation learning in the Morris water maze remains unaffected (Shors *et al.,* 2002). As a fully functioning dentate gyrus is essential for learning and memory (reviewed by Kesner, 2007) the synaptic plasticity provided by neurogenesis may improve the ability of an animal to acquire and retain important spatial information. Mature neurons have a higher threshold for information storage and a more limited capacity to sustain information over time, so the long-term storage capability of younger neurons may be required for some learning tasks. However, the limited capacity of mature neurons to store some learned information may explain why some, but not all, learning tasks are influenced by ablation of hippocampal neurogenesis in rodents (Shors *et al.,* 2002; Raber *et al.,* 2004). As a result, several models for the function of hippocampal neurogenesis in learning have been proposed (Ehninger and Kempermann, 2008):

1. Neurogenesis plays a direct role in the temporary storage of memory and therefore has a transient role. Over time memories become independent of the hippocampus and are processed and stored in other areas such as the neocortex. However, this is unlikely as new neurons persist throughout life and are added to the existing circuits rather than creating a system of continual replacement.
2. Neurogenesis is not directly involved in hippocampus-dependent memory but is more important for refining of existing circuits. This is supported by the continual addition of cells throughout life to already fully functioning circuits.

3. Neurogenesis serves to avoid interference between old and new memories by providing a mechanism that allows the hippocampus to adapt to an increasing number of experiences throughout the life of an animal. The addition of these new neurons therefore increases storage capacity.
4. Neurogenesis plays a role in establishing temporal connectivity between memories and events by allowing new neurons to associate with memories that are developed at the time when the neurons are generated. This theory of strict partitioning would allow the hippocampus to create a time-line of life events and memories.
5. Neurogenesis plays a role in forgetting; the death and replacement of old neurons allows old and unimportant memories to be cleared to make space for new ones. However, this is unlikely as new neurons do not simply replace old ones and neurogenesis shows an age-related decline.

Disruption of forebrain neurogenesis influences olfactory function, but this is limited to olfactory discrimination abilities and not detection or olfactory learning (Gheusi *et al.,* 2000). Additionally, disruption of neurogenesis during pregnancy does not lead to a disruption in maternal behaviour (i.e. grooming and time spent in the nest), but normal pup retrieval is impaired (Feierstein *et al.,* 2010). However, olfactory neurogenesis appears to be important for normal expression of sexual behaviour; females with disrupted olfactory neurogenesis do not respond differentially to males and females, suggesting a role for olfactory neurogenesis in the mediation of an appropriate response to male odours signals (Feierstein *et al.,* 2010). Disruption of neurogenesis therefore does not diminish the ability of females to detect male odour, rather the response to these odours is altered. In support of this, olfactory neurogenesis is required for females to exhibit the normal preference for a dominant male over a subordinate individual and for accurate mate recognition (Mak *et al,* 2007; Oboti *et al.,* 2011). Many different scent components are important for mate recognition and assessment, and pheromonal signatures of individuals are used by female mice to identify and recognise suitable mates. The enhanced plasticity in the neuronal circuitry of the hippocampus and the olfactory system may therefore play an important role in the formation of spatial and odour memories important for conspecific odour detection and discrimination, and ultimately mate choice.

4.1.3.4 Quantification of Neurogenesis

Quantification of neurogenesis typically involves a number of techniques as no single marker identifies the complete process. Ultimately, detection of neurogenesis relies on combining several different labels to measure the two main stages of development: cellular proliferation and the commitment of cells to a neuronal lineage. Cellular proliferation only occurs during the initial stages of neurogenesis and only stem cells are able to contribute to a pool of neuronal precursors, defined by their multipotent potential and ability to self-renew. Expansion of the population of these progenitor cells is governed by the process of repeated entry into the cell cycle. Therefore, the most effective way to identify and quantify cellular proliferation is via analysis of cell cycle activity (reviewed by Kuhn and Peterson, 2008). The DNA replication event that occurs during the S-phase of the cell cycle provides opportunity for integration of a thymidine analogue to quantify replication. A thymidine analogue incorporates not only into the cell during this specific phase, but remains incorporated in the DNA of any progeny of this cell (Fujita *et al.,* 1966; Gratzner, 1982), providing an additional opportunity to track the fate of labelled cells.

A commonly used thymidine analogue is 5-bromo-2-deoxyuridine (BrdU). This compound is incorporated during DNA replication and can be detected using immunohistochemistry even in thick tissue sections, providing accurate labelling of proliferation events (Scharfman *et al.,* 2005). However, there are many problems associated with quantifying cellular proliferation using BrdU (reviewed by von Bohlen Und Halbach, 2007). These include:

1. The requirement for tissue fixation and denaturing of DNA using hydrochloric acid or enzymatic digestion. Even thick sections tissue may be destroyed or damaged.

2. The amount of thymidine analogue incorporated following delivery via a single injection. The small amounts administered are diluted to almost undetectable levels after several rounds of cell division. This often leads to a requirement for multiple injections and thus raises ethical issues as well as increased time and cost (Cameron and McKay, 2001).

3. The incorporation of thymidine analogues into damaged DNA undergoing repair or cells undergoing re-entry to the cell cycle as part of apoptosis (Zucconi and

Giuditta, 2002; Bauer and Patterson, 2005). As a result, BrdU may also label DNA synthesis not directly related to cells undergoing neurogenesis.

4. The lack of synchrony in proliferation of cells within the neurogenic regions of the brain. BrdU labels a distribution of cells during S-phase. As a result, individual cells may only partially overlap the availability of BrdU in the tissue leading to differential uptake between cells and differences in staining intensity within the same cell population (Cameron and McKay, 2001).

5. Physical and behavioural abnormalities as a result of injections (Kolb *et al.*, 1999). This may lead to ethical concerns for the welfare of subjects as well as an unquantifiable influence on the outcome of experiments. The toxic effects of BrdU may also affect stem cell populations in an unknown manner.

6. Alterations in blood flow or permeability of the blood-brain barrier during the experimental period. Variation in BrdU staining may therefore not simply be a result of changes in proliferation rate (reviewed by Gould and Gross, 2002).

7. Changes in cell cycle length as a result of pharmacological or behavioural treatments. Changes in cell cycle length can influence the ability of cells to proliferate, self-renew and differentiate, having implications for any fate-tracking experiments involving BrdU.

8. The lack of specificity in cell labelling (reviewed by Taupin, 2007). All proliferating cells in the region of interest are labelled by BrdU, including those that will eventually differentiate into neurons or supporting cells. As a result BrdU alone cannot be used to quantify neurogenesis specifically.

To circumvent some of these problems, an alternative endogenous marker of cellular proliferation may be used. Unlike BrdU, Ki67, a nuclear protein expressed during the active phases of the cell cycle, does not have any adverse effects on cells and is a reliable marker of mitosis (Brown and Gatter, 2002; Kee *et al.,* 2002). Other endogenous proteins such as doublecortin (DCX) and NeuN, which are expressed by developing and mature neuronal cells respectively (Mullen *et al.*, 1992; Francis *et al.*, 1999), can be used to quantify cells at different stages of neurogenesis. Doublecortin is particularly useful as it is expressed exclusively in migrating neuroblasts and immature neurons (Rao and Shetty, 2004), and NeuN is a soluble nuclear protein expressed during

the later stages of neuron development making it a reliable marker of postmitotic neurons (Lind *et al.*, 2005).

4.1.4 Aims and objectives

Current data suggest that in mice, only odours from dominant males stimulate a significant increase in the generation of new neurons (Mak *et al.,* 2007). However, it is unknown if neurogenesis is influenced by specific scent components such as those that may be important in female assessment and identification of males. Therefore, the overall aim of this study was to identify whether the scent components important for learning of male scents and their locations are also important for stimulation of cellular proliferation and neuron generation in the brains of adult females. To address each of the specific objectives outlined below, the number of proliferating cells in the SVZ and new neurons in the SGZ of the DG was quantified in females exposed to conspecific scents as described. The objectives were to address the following questions:

1. **Does prolonged exposure to male urine stimulate an increase in neurogenesis in females?**

   Soiled male bedding has previously been used to assess the influence of male odour on female neurogenesis (Mak *et al.,* 2007); thus, the first objective of this study was to address the above question. As many of the principal components of conspecific odours important for chemical communication in mice are found in urine, females were exposed to urine from unrelated and unfamiliar females or males of their own strain to determine whether the components of male scent that stimulate neurogenesis are in urine.
2. **Is the male-specific MUP, darcin, required for male urine to stimulate an increase in neurogenesis in females?**

   To assess whether this pheromone plays a role in the stimulation of neurogenesis by exposure to male urine, females were exposed to either male BALB/c urine (that contains negligible levels of naturally produced darcin) or male BALB/c urine with the addition of recombinant darcin (1µg per 1µl of urine).

3. **Is the response to male urine containing recombinant darcin specifically due to the addition of darcin?**

   Recombinant darcin and other r-MUPs were stored in phosphate buffer, so small quantities of this solution were combined with male BALB/c urine when r-darcin was added. Adding r-darcin also increased the overall protein concentration. To ensure the response to male BALB/c urine containing r-darcin was not influenced by the presence of buffer or an overall increase in protein, females were exposed to male BALB/c urine containing buffer or male BALB/c urine containing another novel recombinant MUP, male-specific MUP7, (molecular weight 18645Da). This r-MUP was added to urine at the same concentration as r-darcin (1μg per 1μl urine).

4. **When not in the context of other urinary components, can darcin stimulate an increase in neurogenesis?**

   The darcin protein can stimulate a learned preference for its location (Roberts *et al*, 2012), suggesting this pheromone is a potent stimulator of spatial learning. To determine if this pheromone can stimulate an increase in neurogenesis when not in the context of other urinary components, females were exposed to buffer, r-darcin or r-MUP7, another male-specific MUP.

5. **Is direct contact with male urine required for stimulation of neurogenesis?**

   To assess the importance of direct contact with a scent for stimulation of neurogenesis, females were exposed to male BALB/c urine containing r-darcin that they were either able to contact or unable to contact. In both treatment groups, nesting material was presented within and around a tea strainer. Male urine was added to nesting material outside of the tea strainer in cages where contact with the scent was permitted; urine was only added to the nesting material inside the tea strainer in cages where females were only exposed to the volatile components of male scent.

6. **Does the concentration of darcin in male urine influence the magnitude of an increase in neurogenesis?**

   Results described in Chapter 3 of this thesis show that the amount of darcin is important for female learning of male scent locations; darcin concentration may also be important in the stimulation of female neurogenesis by male scent. To assess how darcin concentration may influence cellular proliferation and neuron number, females were exposed to male BALB/c urine with the addition of one of a range of darcin concentrations similar to those tested in Chapter 3 (0.01μg/μl, 0.1μg/μl,

0.5μg/μl, 1μg/μl, 2μg/μl, 3μg/μl); females exposed to male BALB/c urine with the addition of only buffer served as '0μg/μl' controls.

7. **Is a consistent scent signature of individual identity required for male urine to stimulate an increase in neurogenesis?**

   Urine from male laboratory mice is sufficient to stimulate an increase in neurogenesis, but unlike wild male urine, contains uniform expression of MUPs across individuals of each strain (Cheetham *et al.,* 2009). To establish whether the consistency of the individual scent signature is important for stimulation of neurogenesis by male scent, females were exposed to urine from a single wild stock male or urine from three different unrelated wild-stock males presented in succession. Urine was initially collected from several wild stock males and urine from three males with similar protein and darcin concentration was chosen to ensure the influence of differences in urinary darcin or protein content was minimal.

8. **Does prolonged exposure to male urine stimulate a more robust learned preference for the location of male scent?**

   Male urine can condition a preference for its location following a learning session as brief as 10 minutes (Roberts *et al.,* 2012); extinction of a conditioned place preference occurs rapidly if no scent is encountered in a previously conditioned site (Roberts *et al.,* 2012). As adult hippocampal neurogenesis has been associated with processes important for long-term spatial memory (Snyder *et al.,* 2005), it may be important for promoting more robust learning of male scent locations, preventing extinction of a learned preference even when no scent is encountered. To investigate whether prolonged exposure to male urine over 7 days prevents extinction of a learned preference for the location of male scent, conditioned place preference was assessed in females presented with unfamiliar wild male urine and familiar wild male urine that females were exposed to for 7 days prior to the learning session. A separate group of females was exposed to a familiar male urine stimulus for only 30 minutes prior to the learning session, to ensure any responses observed in females following a 7 day pre-exposure were due to the length of pre-exposure rather than simply due to the effect of familiarity with the urine.

4.2 METHODS

4.2.1 Subjects and urine donors

Subject females were 152 CD-1 laboratory mice. Animals used for neurogenesis experiments (n = 114) were aged 11 weeks (±3 days) at the start of each experiment. Animals used in conditioned place preference tests (n = 38) at the end of the study were aged between 6 and 7 months. Females were bred in house (parents obtained from Harlan UK) and at 3-4 weeks of age were pair housed with a same-sex sibling in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK). Pairs were subsequently moved to larger 45cm x 28cm x 13cm cages at the start of an experiment. In the larger cages all animals were housed with a clear acrylic tunnel (15cm x 5cm), a cardboard shelter (9cm x 11cm x 12cm) and a cardboard tube (4cm x 10cm) during the experiment; these were placed in the same orientation and location within each cage. All subject females were naïve in terms of experience with males or male odours between weaning and the start of an experiment. Females used in conditioned place preference tests were housed in same-sex groups of 4 in 45cm x 28cm x 13cm cages (MB1, North Kent Plastics Ltd., UK).

Urine donors consisted of: 10 adult male and 12 adult female CD-1 mice, 25 adult male BALB/c laboratory mice and 9 adult male wild stock mice bred within the laboratory. All animals used for urine collection were between 6 and 12 months of age. Male and female CD-1 donors and male BALB/c donors were housed in same-sex pairs in 48 cm x 11.5 cm x 12 cm cages (M3, North Kent Plastics Ltd., UK); wild-derived males were housed singly in 48cm x 11.5cm x 12cm cages (M3, North Kent Plastics Ltd., UK). Throughout experiments all subject and urine donor animals were maintained on a reversed 12:12 light cycle (lights off at 08:00h) and on Corn Cob Absorb 10/14 substrate with paper wool bedding material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, MO, USA). Cardboard tubes were provided to all urine donor animals for home cage enrichment.

4.2.2 Stimulus scents

Urine was collected from donors up to five days before the start of an experiment. Donors were confined above a cage on a mesh grid for a maximum of 2 hours and cages were checked for urine every 30 minutes. If urine was present it was

collected immediately and stored at -20°C until use. Urine from individuals of each laboratory strain was combined into same-sex, same-strain pools. When assessing female neurogenesis in response to stimulus odours, recombinant darcin and other r-MUPs were tested alone, (1µg in 1µl buffer) or added to male BALB/c urine (1µg to 1µl urine unless stated otherwise) to mimic the natural concentration of darcin observed in C57BL/6 males (approximately 10-14% of total MUP) and in wild male mice (Armstrong *et al.,* 2005; Cheetham *et al*, 2009). All female subjects were able to directly contact the odour stimulus excluding one group used to assess the response to only the airborne volatile components of scents. In this treatment the nesting material containing the stimulus odour was placed within mesh tea strainers, thus preventing direct contact. Where the response to wild male urine was assessed, females were exposed to urine from the same individual wild-stock male for the full 7 days of the experiment or to urine from three different wild-stock males presented in succession.

4.2.3 Measurement of urinary protein and darcin concentration

Protein concentrations were determined using the Coomassie Plus protein assay reagent kit from Perbio Science UK Ltd. (Northumberland, UK) following methods described by Cheetham *et al.* (2009) and discussed previously in section 2.2.5. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide) was performed as described by Laemmli (1970) and discussed previously in section 2.4.6.

4.2.4 Expression and purification of recombinant MUPs

Recombinant MUPs were expressed and purified by Lynn McLean (Protein Function Group, University of Liverpool, UK) using methods described by Roberts *et al* (2010) and discussed previously in section 3.2.6.

4.2.5 Experimental procedure

Procedures involved in this study included those conducted under Home Office license (i.e. administration of anaesthetic via intraperitoneal injection and transcardial perfusion) under Project License 40/3492 and Personal License 40/9790 (Animals in

Scientific Procedures Act, 1986). In addition, non-invasive standard husbandry and handling procedures that did not involve any pain, suffering, distress or lasting harm were used. The work followed national and international best practice guidelines and was approved by the University of Liverpool Animal Welfare Committee.

All neurogenesis experiments lasted 7 days and began when subjects were aged 11 weeks (±3 days). All female subjects were pair housed with same-sex siblings and three pairs were assigned to each stimulus treatment (n=6/group). Assignment of female pairs from the same litter was balanced across treatments (i.e. each treatment group consisted of three pairs from three different litters). Mead's Resource Equation (Kirkwood and Hubrecht, 2010) was used to determine appropriate sample sizes for treatment groups; allowances for additional complications such as imperfect perfusions were also taken into account. Stimulus urine or buffer containing r-MUPs was added to clean nesting material (250µl urine or buffer solution on 7g of nesting material) and placed into the home cages of subject pairs. Stimulus odours were refreshed every other day during the experiment by removing the old nesting material and replacing it with clean nesting material containing 250µl of fresh stimulus odour. Where direct contact with a stimulus odour was controlled, nesting material was presented within and around a 55mm tea strainer (mesh holes 1mm x 1mm). Male urine was added to nesting material outside of the tea strainer in cages where contact with the scent was permitted; male urine was only added to the nesting material inside the tea strainer in cages where females were to be exposed to only the volatile components of the male urine. On the final morning of the experiment animals were removed individually from cages and anaesthetised with gaseous halothane followed by an intraperitoneal injection of a terminal dose of Euthatal (350µl, 1:10 dilution of pentobarbital sodium solution, JM Loveridge Plc, Southampton). Whilst under terminal deep anaesthesia animals were perfused transcardially with approximately 20ml phosphate buffer (PB) followed by 30ml fixative (4% paraformaldehyde, 0.2% picric acid in 100mM PB) using an *in vivo* perfusion system (AutoMate Scientific, Berkeley, CA). Animals were anaesthetised in a procedures room separate from where the rest of the colony was housed and perfusions took place underneath a fume hood in a separate laboratory. Brains were then immediately dissected free and stored in fixative for four hours at 4^{o}C before being transferred to cryoprotectant (25% sucrose and 0.01% sodium azide in 100mM PB) for storage at 4^{o}C.

4.2.6 Tissue processing

The olfactory bulbs and brain stem were dissected free from brains and 50μm coronal sections collected serially between coordinates Bregma +1.94mm and -4.04mm (Franklin and Paxinos, 2008) of the remaining tissue using a freeze microtome. This ensured all tissue in the regions of interest (lateral ventricles and hippocampus) was processed. Six sets of sections were collected free floating in a 24-well multi-well plate. Each set contained approximately 20 complete sections that were stored in cryoprotectant (30% sucrose, 1% polyvinylpyrrolidone and 30% ethyleneglycol in 100mM PB) at -20°C until immunohistochemical processing.

4.2.7 Immunohistochemistry

All tissue sections were washed 3 x 10 mins in 10mM phosphate buffered saline (PBS) to remove any cryoprotectant residue. They were subsequently incubated overnight in the dark at 4°C with primary antibodies diluted in Triton-X solution (0.3% in 10mM PBS), a detergent that aids penetration of the antibody into tissue. Antibody solutions used were rabbit polyclonal anti-Ki67 (1:100, Vector Laboratories, California), to detect proliferating cells in the SVZ in forebrain sections, or goat polyclonal anti-DCX (1:100, Santa Cruz Biotech, Texas), to detect immature neurons in the SGZ in hippocampal sections. These antibodies were chosen for two main reasons. Firstly, dissection of complete and intact olfactory bulbs from subjects following perfusion proved difficult due to a small number of imperfect perfusions and limited expertise in the fine dissection technique required to remove the olfactory bulbs intact; intact olfactory bulbs were retrieved from only 78 (68%) animals. This meant quantification of cells at the late stages of neurogenesis (i.e. immature neurons) in the olfactory bulbs was not possible for 32% of subjects. Cellular proliferation was therefore measured in the SVZ of the forebrain as an alternative measure of early neurogenesis in the region that provides newly generated cells to the olfactory system. Secondly, initial problems with effective and reliable staining of proliferating cells in the dentate gyrus persisted throughout the experiment. This may have been due to a number of factors, including low proliferation levels in this region or problems with the particular primary or secondary antibodies chosen. Despite several brands, combinations and antibody dilutions being tested, no improvement in visualisation of proliferating cells occurred.

Immature neurons were therefore quantified as a measure of neurogenesis in the hippocampus.

Following incubation with primary antibodies, tissue was washed in PBS, which was followed by 1 hour incubation with secondary antibodies at room temperature in the dark. Forebrain sections were incubated with red fluorophore-conjugated secondary antibody raised in donkey (Alexa Fluor® 594-AffiniPure (1:400), Stratech Scientific Ltd., Newmarket); hippocampal sections were incubated with green fluorophore-conjugated antibody raised in rabbit (Alexa Fluor® 488-AffiniPure (1:400), Stratech Scientific Ltd., Newmarket). After rinsing with PBS, sections were mounted onto microscope slides (8 per slide) coated with a chrome-alum gelatin (CAG) solution. After drying overnight, tissue was coverslipped under anti-fade medium (Vectorshield®, Vector Laboratories, Peterborough). Optimal working concentrations of all antibodies were determined in earlier tests on spare tissue by applying serial dilutions prepared in 0.3% Triton-X solution.

Immunostaining was examined under an epi-fluorescence microscope (Zeiss AxioImager. M1); images were captured by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu photonics, Welwyn Garden City, Hertfordshire, UK) and an image analysis program (Axiovision LE 4.8.2., Zeiss Imaging Systems). Immuno-positive cells were quantified in the left hemisphere of all brains. Due to tissue thickness, the Z-stack multi-acquisition function was used to capture sets of approximately 30 images through each section. Positively stained cells were counted automatically on photomicrographs using the 'Analyze Particles' tool in ImageJ version 1.4 (http://rsb.info.nih.gov/ij/). The threshold range was set to 65-165, although in some cases the lower threshold was adjusted upwards after manual inspection to avoid miscounting. Subjective cell counts were also performed manually using the same photomicrographs to ensure automatic counts provided by ImageJ were accurate. After excluding top and bottom images to avoid counting cells that were incomplete, Ki67- or DCX-immunopositive cells were counted in every tenth Z-stack image to avoid repeat sampling of the same cell in adjacent images. This process was repeated for each of the 8 sections mounted per slide. The total number of stained cells counted per slide was multiplied by 2 to account for both brain hemispheres and then by 6 to account for the serial collection of sets during sectioning. This final number was used as an estimate of the total number of positively stained cells within the entire SVZ or SGZ.

4.2.8 Conditioned place preference tests

Following a single 10 minute scent exposure, extinction of a conditioned place preference occurs rapidly if no scent is encountered in a previously conditioned site (Roberts *et al.,* 2012). To investigate whether prolonged exposure to male urine over 7 days prevents extinction of a learned preferences for the location of male scent, conditioned place preference was assessed in females presented with unfamiliar wild male urine and familiar wild male urine that females were exposed to for 7 days prior to the learning session. A separate group of females was exposed to the familiar male urine stimulus for only 30 minutes prior to the learning session, to ensure any responses observed in females following a 7 day pre-exposure were due to the length of pre-exposure rather than simply due to the effect of familiarity. Conditioned place preference tests comprising four locations and therefore four stimuli were conducted following methods described in Chapter 3, section 3.2.3. After confirming no location bias (no urine), female subjects were presented with the two scent stimuli (unfamiliar and familiar wild male urine) and two control stimuli (both ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours later with no urine present (24h memory). Two additional modifications to place preference tests were included: (1) prior to the learning session, females were exposed to urine from a single wild-stock male for either 30-min or 7d and urine from the same male was subsequently presented during the learning session along with a second, unfamiliar urine stimulus and two control stimuli; (2) the response of females in the absence of scent was assessed on two successive days rather than in a single 24-hour memory test. This allowed quantification of the extinction response.

4.2.9 Data Analysis

All statistical tests were carried out using the SPSS software package, version 20. Due to small treatment group sizes (n = 6/group) and non-normal data distributions (assessed by Kolmogorov Smirnov and Shapiro Wilks tests, $p < 0.05$), differences in Ki67-positive and DCX-positive cell counts between individual treatment groups were examined using non-parametric Mann-Whitney U tests (one-tailed exact tests to assess greater number of immunopositive cells in females exposed to urine stimuli versus in females exposed to control stimuli). Where differences in cell counts between females

exposed to different urine stimuli were of interest, further comparisons between specific treatment groups were also made. A Spearman's rank correlation with untransformed data tested for a relationship between the number of immunopositive cells and the concentration of darcin present in urine.

In conditioned place preference tests, females were expected to show significantly greater attraction to a urine stimulus compared to the water controls during learning sessions and to spend more time in the learned location of a urine stimulus if the urine stimulated a conditioned place preference (Chapter 3). For both tests, the difference between time spent in the location where the urine stimulus was presented during the learning session and mean time spent in the control locations was analysed. As data distribution approximated normality (assessed by Kolmogorov Smirnov and Shapiro Wilks tests, $p > 0.05$), repeated measures ANOVAs were used to test whether females spent significantly longer in the location of the urine compared to the location of the control and whether there was an overall effect of scent pre-exposure on female responses.

4.3 RESULTS

4.3.1 Does prolonged exposure to male urine stimulate an increase in neurogenesis in females?

Odours from dominant male mice stimulate an increase neurogenesis in the hippocampus and olfactory bulb of females; this increase is time dependent and occurs after seven days of exposure to soiled male bedding or an intact male (Mak *et al.*, 2007; Larsen *et al.,* 2008). As many of the principal components of odours important in chemical communication are found in urine, females were exposed to urine from unrelated and unfamiliar females or males of their own strain to determine whether male urine stimulates an increase in neurogenesis. Compared to females exposed to water, there were significantly more Ki67-positive cells in the SVZ of females exposed to male, but not female urine (Mann Whitney U test: Male urine: U = 5.00, Z = -2.08, p = 0.02; Female urine: U = 11.00, Z = -1.12, p = 0.16; **Figures 4.1a & 4.2**). In addition, the number of hippocampal DCX-positive cells was significantly greater in females exposed to male urine when compared to females exposed to water (Mann Whitney U test: U = 3.00, Z = -2.40, p = 0.008; **Figures 4.1b & 4.3**). Surprisingly, the number of DCX-positive cells in the hippocampus was also significantly greater in females exposed to urine from unfamiliar and unrelated females of their own strain (Mann Whitney U test: U = 7.00, Z = -1.76, p = 0.047). Further, contrary to expectations, the number of positively stained cells in both the SVZ and hippocampus of females exposed to female urine only showed a tendency to be different from staining in females exposed to male urine containing darcin (Mann Whitney U test: Ki67: U = 11.00, Z = -1.12, p = 0.16; DCX: U = 9.00, Z = -1.44, p = 0.09). To determine whether this result and the increase in DCX-positive cells in females exposed to female urine was typical for subjects in this study, an additional group of females were exposed to female CD-1 urine in a subsequent experiment (results are described in section 4.3.3).

4.3.2 Is the male-specific MUP, darcin, required for male urine to stimulate an increase in neurogenesis in females?

The male pheromone, darcin, is expressed in the urine of male wild mice and in the urine of the majority of laboratory strains. This male-specific MUP is important for

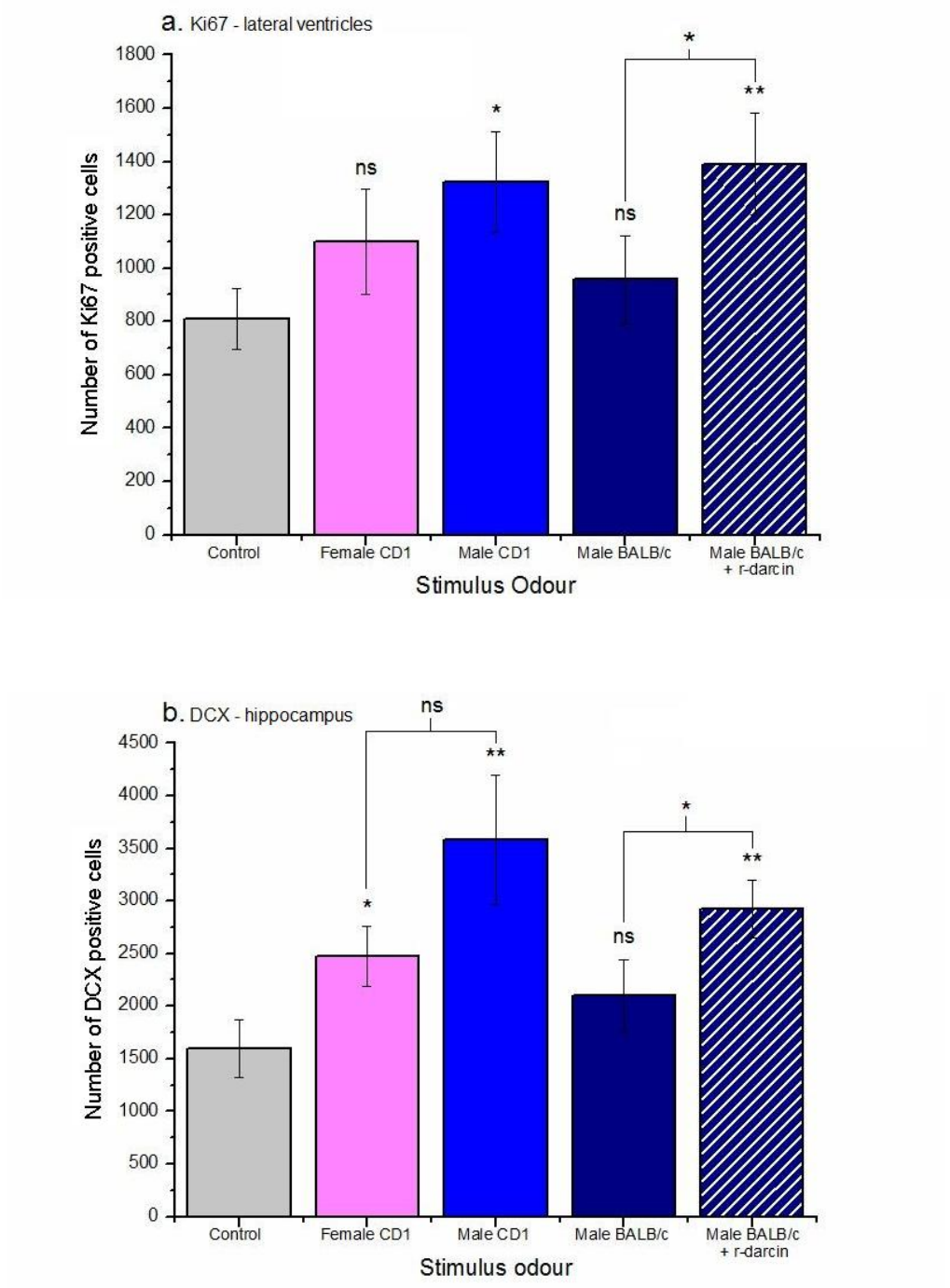

Figure 4.1 Proliferating cells and immature neurons in females exposed to male or female urine.

Number of (a) Ki67-positive proliferating cells in the SVZ and (b) DCX-positive immature neurons in the dentate gyrus of females exposed to water (*grey*), female CD-1 urine (*pink*), male CD-1 urine (*light blue*), male BALB/c urine (*dark blue*) or male BALB/c urine + r-darcin (*dark blue hatched*). A greater number of immunopositive cells in females exposed to urine versus in females exposed to a water control was assessed by Mann-Whitney U tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

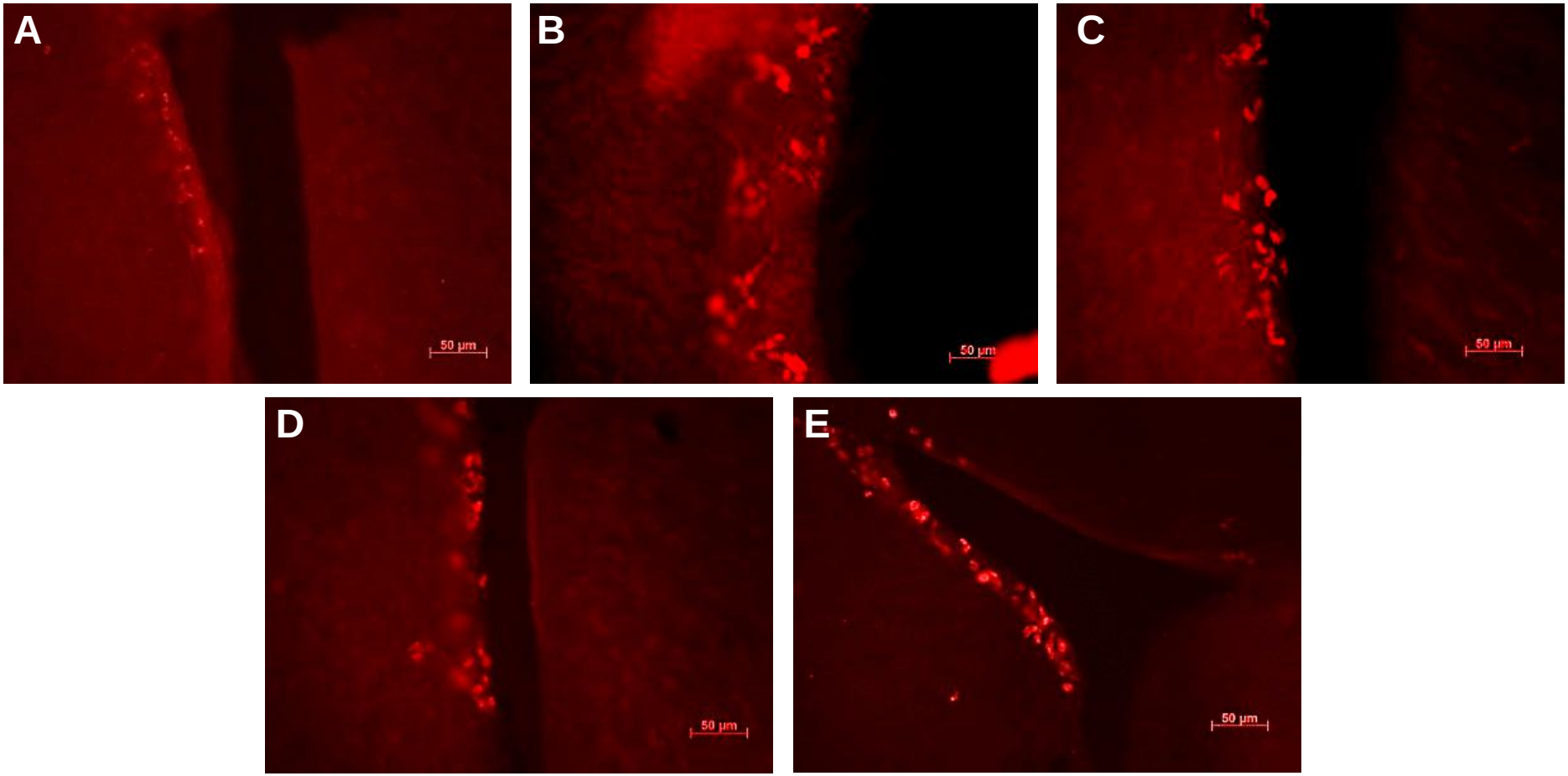

Figure 4.2 Representative micrographs of Ki67-positive cells.
Ki67-positive cells in the SVZ of the lateral ventricles in females exposed to (A) water (ddH_2O), (B) female CD-1 urine, (C) male CD-1 urine, (D) male BALB/c urine or (E) male BALB/c urine + 1µg/µl r-darcin.

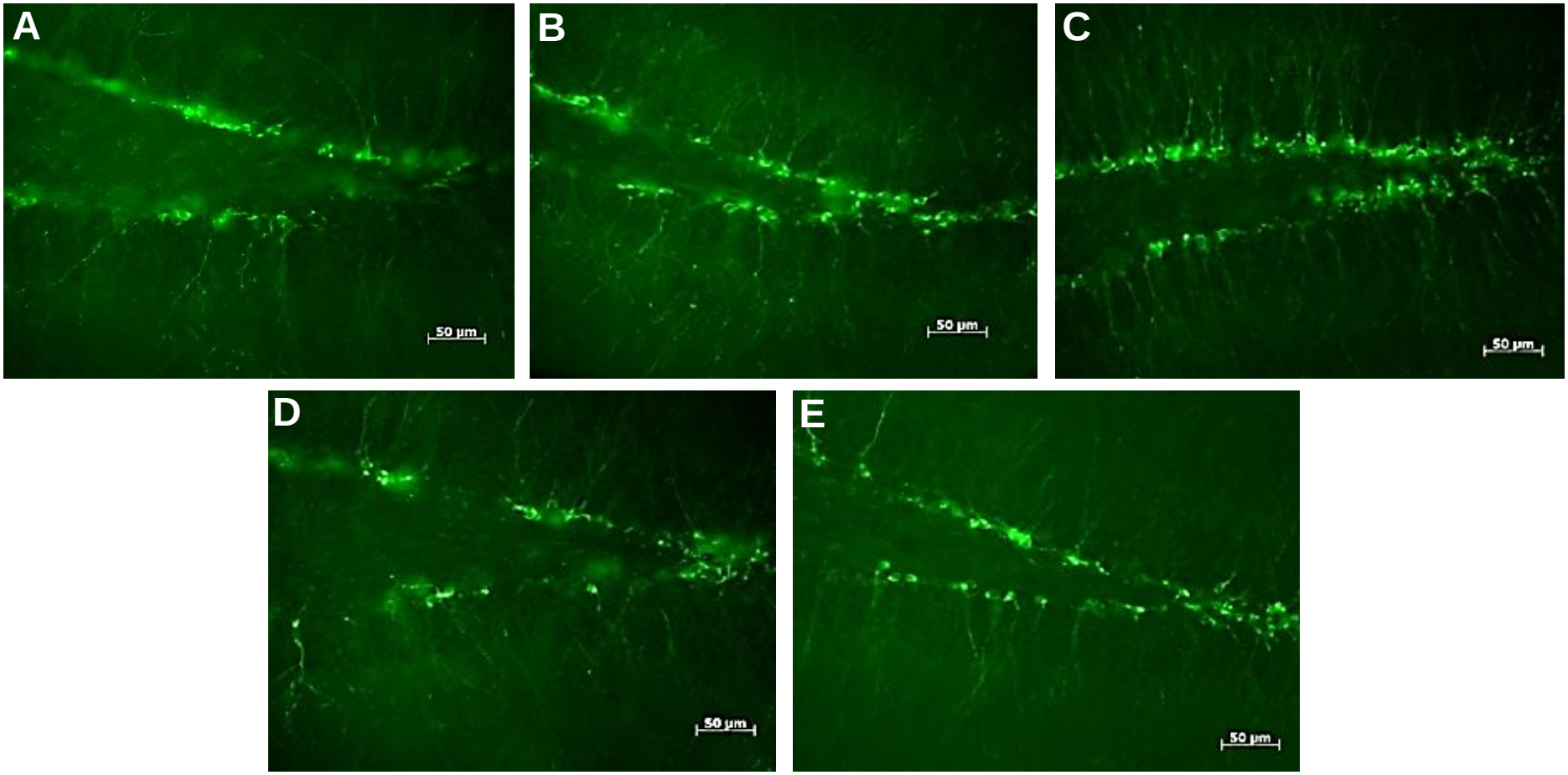

Figure 4.3 Representative micrographs of DCX-positive cells.
DCX-positive cells in the SGZ of the dentate gyrus (hippocampus) in females exposed to (A) water (ddH_2O), (B) female CD-1 urine, (C) male CD-1 urine, (D) male BALB/c urine or (E) male BALB/c urine + 1µg/µl r-darcin.

female attraction to male scent (Roberts *et al.,* 2010), and can condition a learned preference for its location (Roberts *et al.,* 2012). Male BALB/c laboratory mice naturally produce negligible amounts of urinary darcin due to the removal of sexual selection pressures as a result of inbreeding over many generations (Cheetham e*t al.,* 2009). To test whether this pheromone plays a role in stimulation of neurogenesis by male urine, females were exposed to male BALB/c urine or male BALB/c urine with the addition of recombinant darcin (1μg per 1μl of urine). Compared to females exposed to water, there were significantly more Ki67-positive cells in the SVZ of females exposed to male BALB/c urine with added r-darcin (Mann-Whitney U test: U = 3.50, Z = -2.33, p = 0.009; **Figures 4.1a & 4.2**), but not in females exposed to male BALB/c urine alone (Mann-Whitney U test: U = 9.00, Z = -1.44, p = 0.09; **Figures 4.1b & 4.2**). There was also significant difference between these groups cells (Mann-Whitney U test: U = 5.00, Z = -2.08, p = 0.02). Similarly, the number of DCX-positive cells in the hippocampus was significantly higher in females exposed to male BALB/c urine containing r-darcin (Mann Whitney U test: U = 3.00, Z = -2.40, p = 0.008; **Figures 4.1b & 4.3**), but not in females exposed to BALB/c male urine (Mann Whitney U test: U = 11.00, Z = -1.12, p = 0.16; **Figures 4.1b & 4.3**); there was a significant difference between these two groups (Mann Whitney U test: U = 7.00, Z = -1.76, p = 0.047).

4.3.3 Is the response to male urine containing recombinant darcin specifically due to the addition of darcin?

Recombinant darcin and other r-MUPs are stored in phosphate buffer, so small quantities of this solution are combined with male BALB/c urine when r-darcin is added. Adding r-darcin also increases the overall protein concentration of male urine. To ensure responses to male BALB/c urine containing r-darcin were not influenced by the buffer or an overall increase in protein, females were exposed to male BALB/c urine containing buffer or male BALB/c urine with the addition of another r-MUP, male-specific MUP7, added at the same concentration as r-darcin (1μg per 1μl urine). A group of females exposed to male BALB/c urine with the addition of r-darcin served as positive controls. Additionally, six females were exposed to female CD-1 urine, a treatment repeated to determine whether results described in section 4.3.1 were typical for females in this study. Compared to females exposed to water, the number of Ki67-

positive cells in the SVZ was not significantly different in females exposed to any of the different stimulus scents (Mann Whitney U test: Female urine: U = 17.00, Z = -0.16, p = 0.47; BALB/c male + buffer: U = 17.00, Z = -0.16, p = 0.47; BALB/c male + r-MUP7: U = 16.00, Z = -0.32, p = 0.41; **Figures 4.4a & 4.5**). Similarly, compared to females exposed to water, the number of DCX-positive cells in the hippocampus was not significantly different in females exposed to any of the stimuli (Mann Whitney U test: Female urine: U = 17.00, Z = -0.16, p = 0.47; BALB/c male + buffer: U = 16.00, Z = -0.32, p = 0.41; BALB/c male + r-MUP7: U = 13.00, Z = -0.80, p = 0.24; **Figures 4.4b & 4.6**).

However, in a previous experiment (results described in section 4.3.1), this difference was significant. To assess the overall difference in the number of DCX-positive cells in females exposed to female urine compared to females exposed to water, a non-parametric rank-based two-way ANOVA was used (Meddis, 1984). This allowed the different levels of positive staining across the two experiments to be taken into account. Overall, DCX-positive staining was not significantly greater in females exposed to female urine compared to females exposed to water (two-way ANOVA: $\chi^2(1) = 0.33$, p = 0.56); further, despite there being a significant difference in positive staining between the two experiments ($\chi^2(1) = 17.28$, $p < 0.001$), there was no interaction between experiment and stimulus odour (interaction between experiment and stimulus: $\chi^2(1) = 0.48$, p = 0.49). Overall, DCX-positive staining did not differ in females exposed to female urine compared to females exposed to water. In addition, females exposed to male BALB/c urine with the addition of r-darcin (the positive control in this experiment) had significantly more positively stained cells in both the SVZ and hippocampus than females exposed to female urine (Mann Whitney U test: Ki67: U = 3.00, Z = -2.40, p = 0.008; DCX: U = 0.00, Z = -2.88, $p < 0.001$).

4.3.4 When not in the context of other urinary components, can darcin stimulate an increase in neurogenesis?

The darcin protein can stimulate a learned preference for its location (Roberts *et al*, 2012), suggesting this pheromone is a potent stimulator of spatial learning. To determine if this pheromone can also stimulate an increase in neurogenesis, females were exposed to phosphate buffer, buffer containing 1μg/μl r-darcin, or buffer

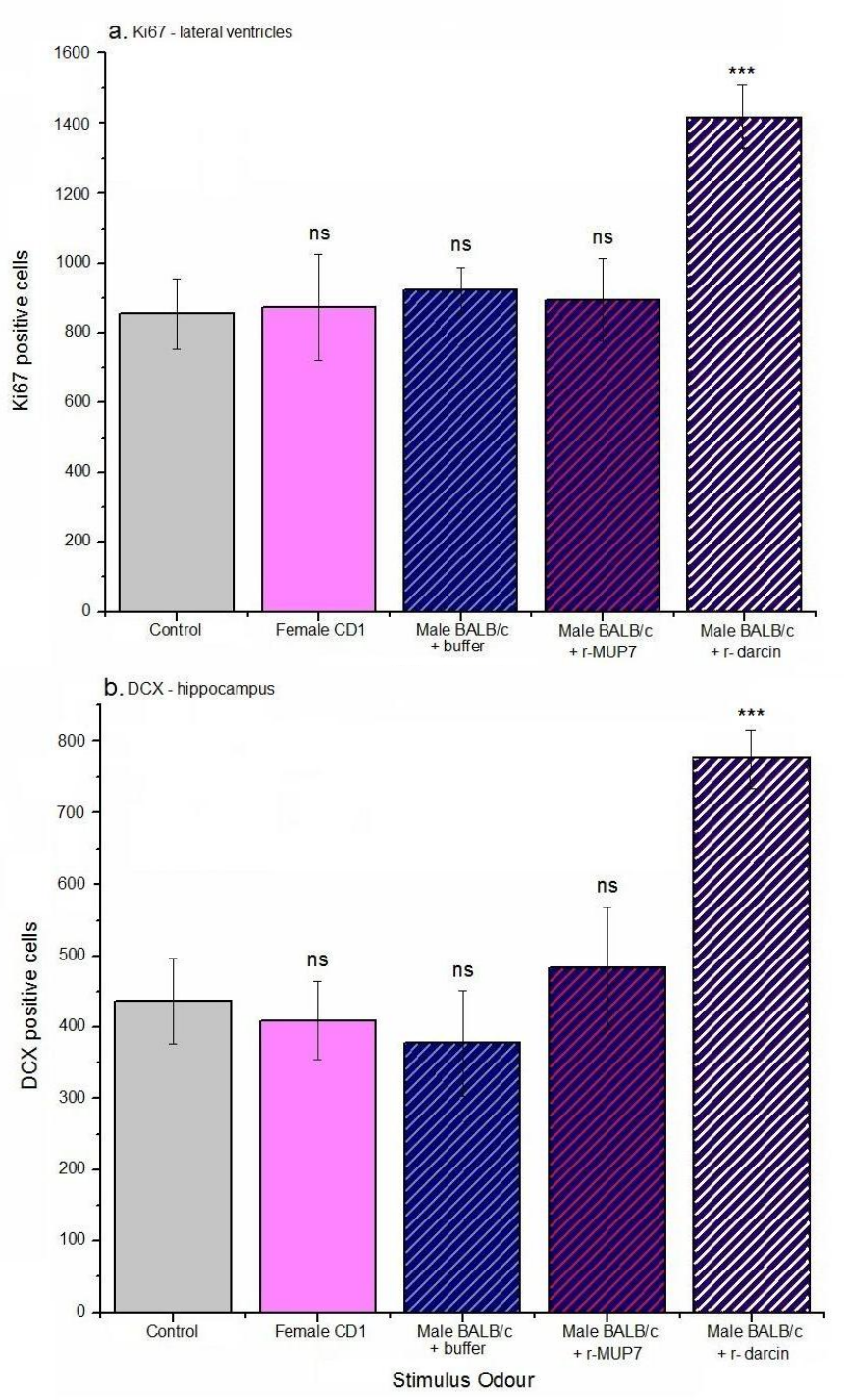

Figure 4.4 Proliferating cells and immature neurons in females exposed to female urine or male BALB/c urine.

Number of (a) Ki67-positive proliferating cells in the SVZ and (b) DCX-positive immature neurons in the dentate gyrus of females exposed to water (*grey*), female CD-1 urine (*pink*), male BALB/c urine + buffer (*grey stripes*), male BALB/c urine + r-MUP7 (*red stripes),* or male BALB/c urine + r-darcin (*white stripes*). A greater number of immunopositive cells in females exposed to urine versus in females exposed to a water control was assessed by Mann-Whitney U tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

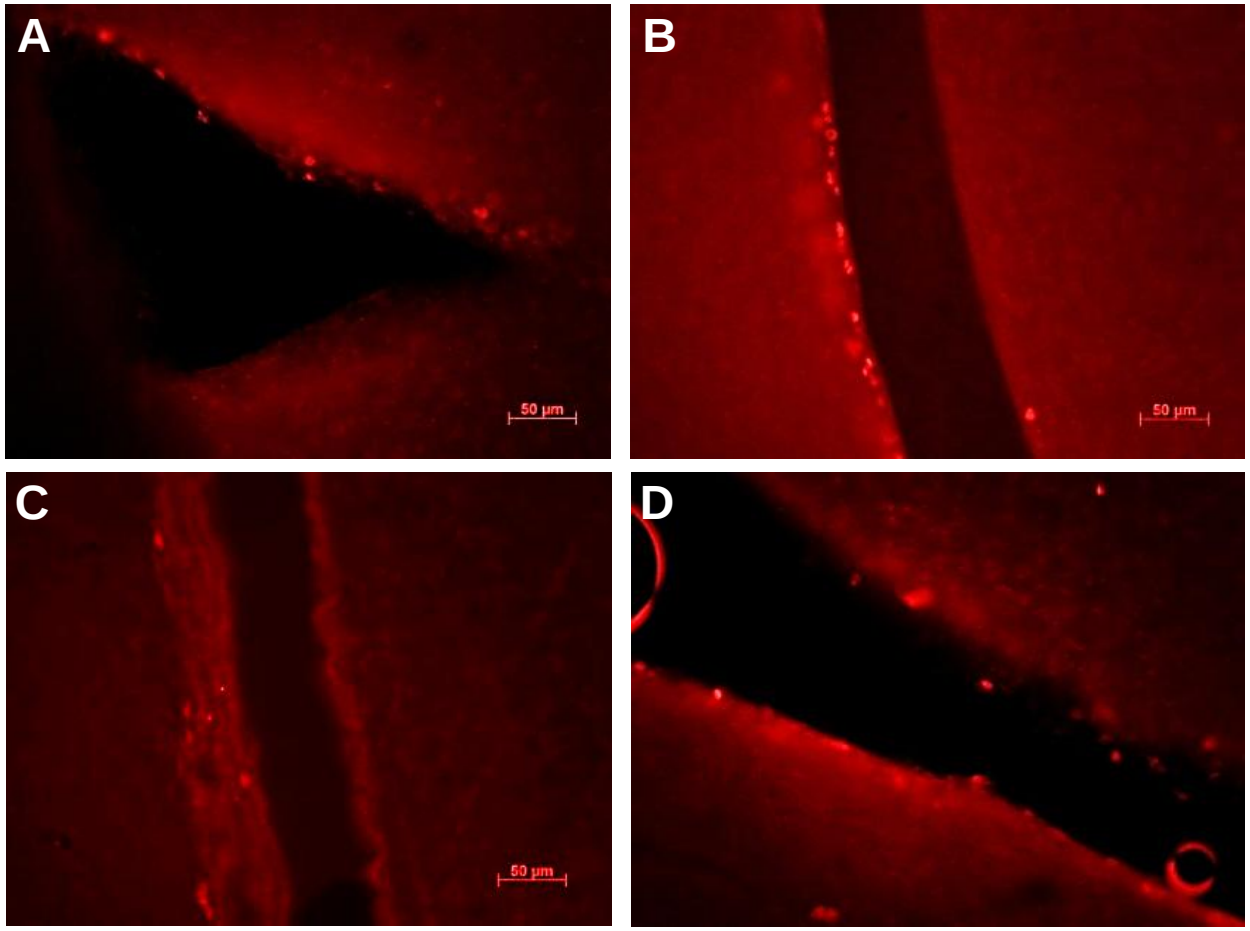

Figure 4.5 Representative micrographs of Ki67-positive cells.
Ki67-positive cells in the SVZ of the lateral ventricles in females exposed to (A) water, (B) female CD-1 urine, (C) male BALB/c urine + buffer or (D) male BALB/c urine + 1µg/µl r-MUP7.

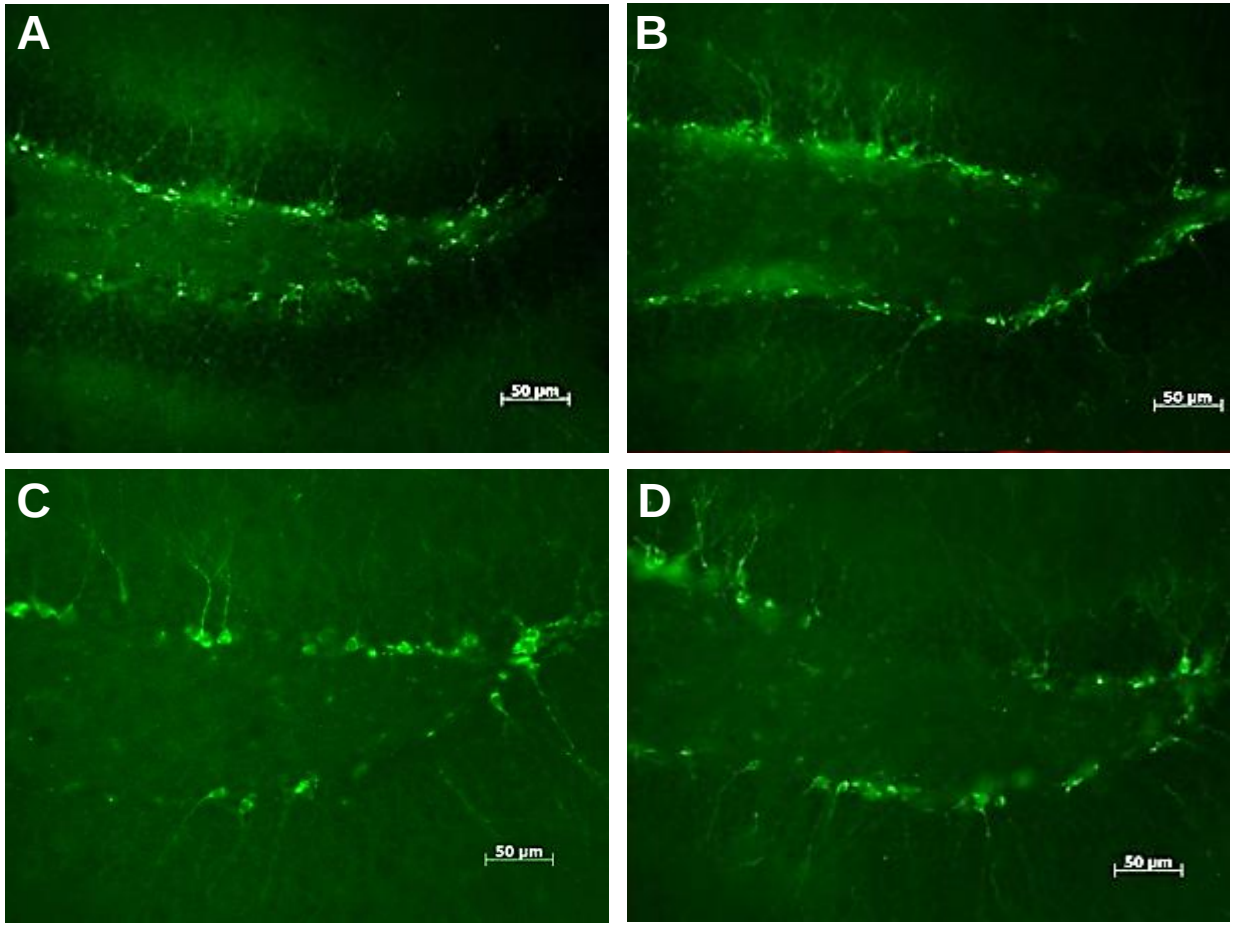

Figure 4.6 Representative micrographs of DCX-positive cells.
Ki67-positive cells in the SGZ of the dentate gyrus (hippocampus) in females exposed to (A) water, (B) female CD-1 urine, (C) male BALB/c urine + buffer or (D) male BALB/c urine + 1µg/µl r-MUP7.

containing 1μg/μl of r-MUP7. When compared to females exposed to a buffer control, a significantly greater number of Ki67-positive proliferating cells was found in the SVZ of females exposed to buffer containing r-darcin (Mann Whitney U test: U = 5.00, Z = -2.08, p = 0.02; **Figures 4.7a & 4.8**), but not in females exposed to buffer containing r-MUP7 (Mann Whitney U test: U = 11.00, Z = -1.12, p = 0.16; **Figures 4.7a & 4.8**).Similarly, females exposed to buffer containing r-darcin, but not r-MUP7, had significantly more DCX-positive immature neurons in the hippocampus than females exposed to buffer (Mann Whitney U test: r-darcin: U = 2.00, Z = -2.57, p = 0.004; r-18645: U = 14.00, Z = -0.64, p = 0.29; **Figures 4.7b & 4.9**).

4.3.5 Is direct contact with male urine required for stimulation of neurogenesis?

Female attraction to spend time near male scent requires prior contact with the scent of an individual male (Ramm *et al.,* 2008), and contact with a male scent is thought to be rewarding for females (Martinez-Garcia *et al.,* 2009). Airborne volatiles are likely to be important in alerting females to the presence of male scent, but learning of the individual-specific volatile profile of an individual male is only possible through prior contact with the male-specific pheromone, darcin (Roberts *et al*, 2012). Direct contact with a scent may therefore be required for male scent to stimulate neurogenesis. To assess the importance of direct contact for stimulation of neurogenesis, females were exposed to male BALB/c urine with the addition of r-darcin (1μg/μl) that they were either able to contact or unable to contact. In both treatment groups, nesting material was presented within and around a tea strainer. Male urine was added to nesting material outside of the tea strainer in cages where contact with the scent was permitted; urine was only added to the nesting material inside the tea strainer in cages where females were only exposed to the volatile components of male scent. Compared to animals exposed to a buffer control, the number of Ki67-positive cells in the SVZ was only significantly higher in females that were able to contact the male urine (Mann Whitney U test: Contact: U = 4.00, Z = -2.24, p = 0.01; No contact: U = 13.00, Z = -0.80, p = 0.24; **Figures 4.7a & 4.8**). There was also a significant difference in the number Ki67-positive proliferating cells between these two groups (Mann Whitney U test: U = 5.00, Z = -2.08, p = 0.02). Similarly to Ki67 staining, a significantly greater

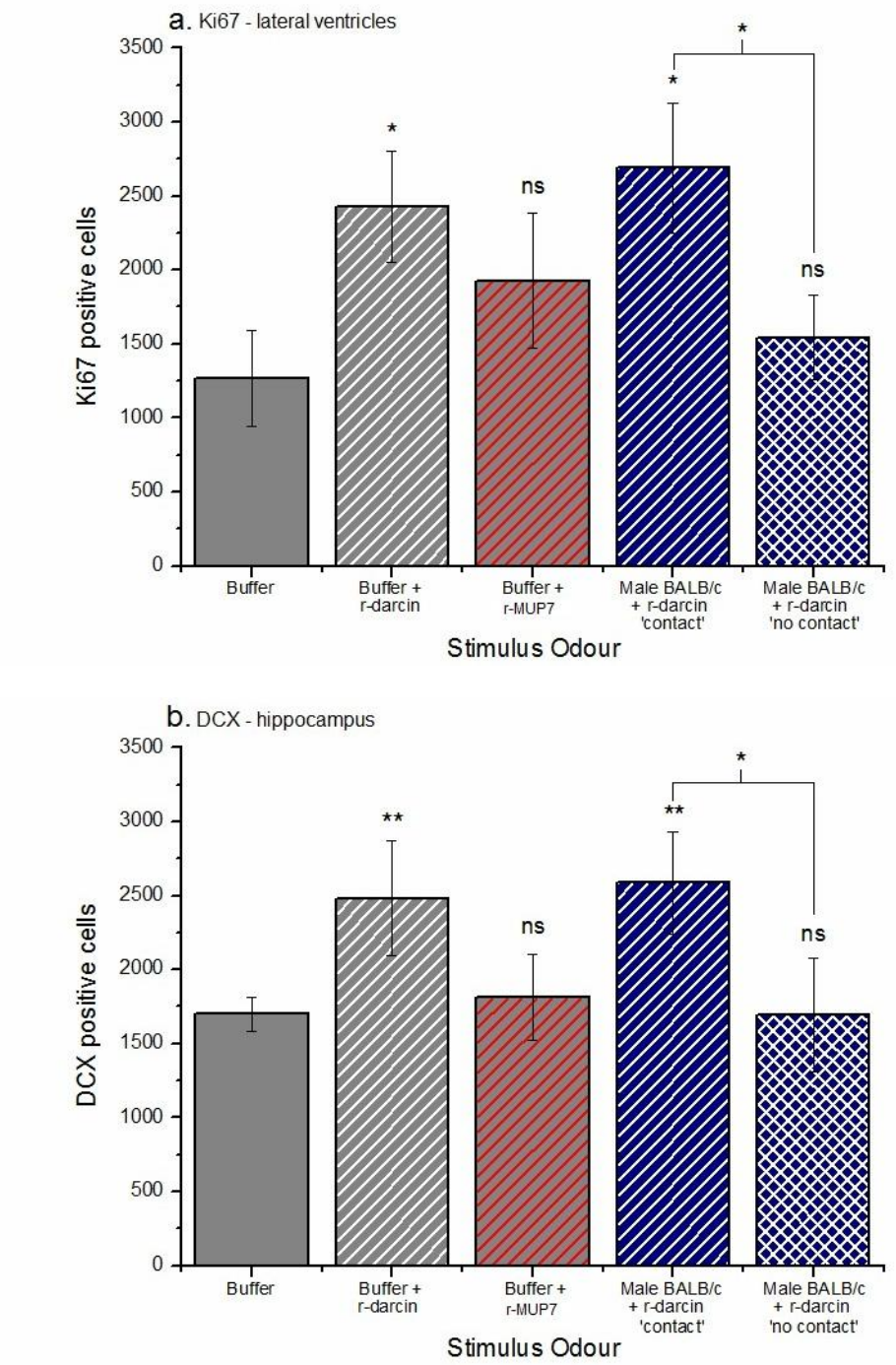

Figure 4.7 Proliferating cells and immature neurons in females exposed to r-MUPs or male BALB/c urine containing r-darcin with or without direct contact. Number of (a) Ki67-positive proliferating cells in the SVZ and (b) DCX-positive immature neurons in the dentate gyrus of females exposed to buffer (*grey*), buffer + r-darcin (*grey and white stripes*), buffer + r-MUP7 (*grey and red stripes),* male BALB/c urine + r-darcin with contact (*blue and white stripes*) or male BALB/c urine + r-darcin without contact (*blue and white cross-hatch*). A greater number of immunopositive cells in females exposed to urine or buffer containing r-MUP versus in females exposed to a buffer control was assessed by Mann-Whitney U tests:* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean number of stained cells ± SEM.

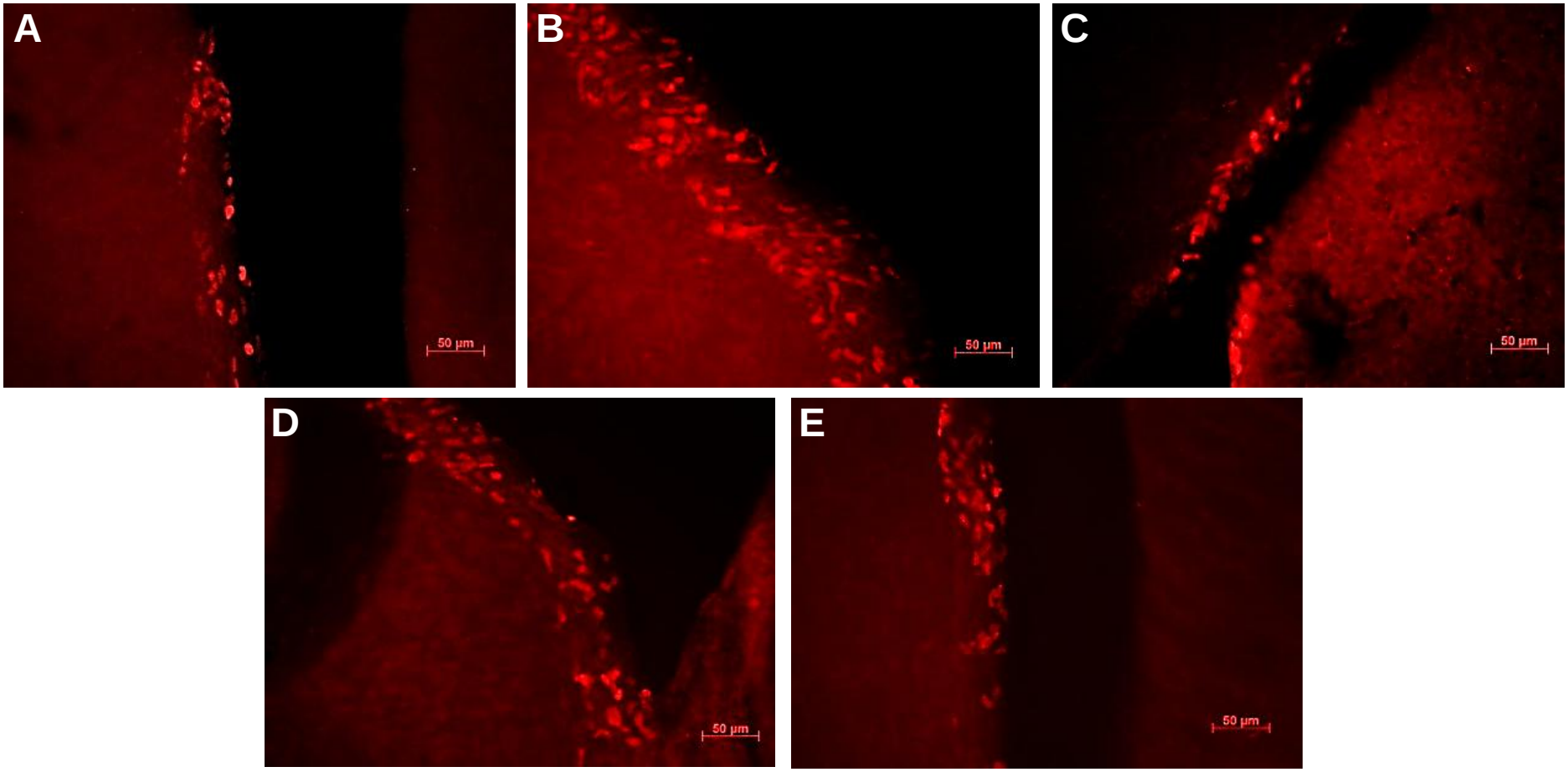

Figure 4.8 Representative micrographs of Ki67-positive cells.
Ki67-positive cells in the SVZ of the lateral ventricles in females exposed to (A) phosphate buffer, (B) buffer + 1µg/µl r-darcin, (C) buffer + 1µg/µl r-MUP7, (D) male BALB/c urine + 1µg/µl r-darcin with contact or (E) male BALB/c urine + 1µg/µl r-darcin with no direct contact.

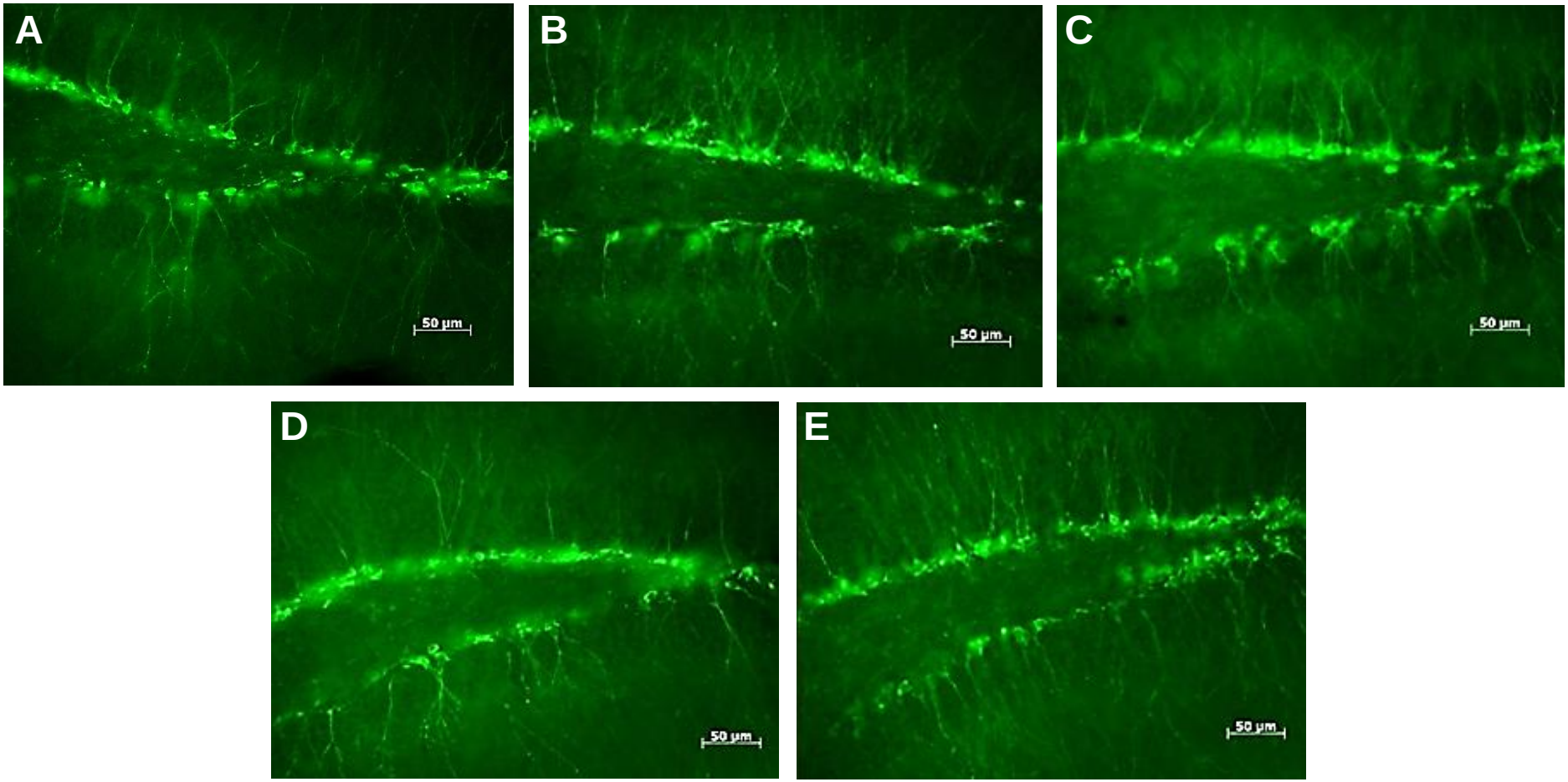

Figure 4.9 Representative micrographs of DCX-positive cells.

DCX-positive cells in the SGZ of the dentate gyrus (hippocampus) in females exposed to (A) phosphate buffer, (B) buffer + 1µg/µl r-darcin, (C) buffer + 1µg/µl r-MUP7, (D) male BALB/c urine + 1µg/µl r-darcin with contact or (E) male BALB/c urine + 1µg/µl r-darcin with no contact.

number of DCX-positive neurons was only present in the hippocampus of females that were able to make direct contact with the scent (Mann Whitney U test: Contact: U = 2.00, Z = -2.56, p = 0.004; No contact: DCX: U = 11.00, Z = -1.12, p = 0.15; **Figures 4.7b & 4.9**); there was a significant difference in positively stained cells between these two treatments (Mann Whitney U test: U = 5.00, Z = -2.08, p = 0.02).

4.3.6 Does the concentration of darcin in male urine influence the magnitude of an increase in neurogenesis?

Results described in Chapter 3 show the amount of darcin is important for female learned preferences for male scent locations; further, the threshold concentration of darcin required for learning to occur was estimated to be between 0.05 and 0.1μg/μl. To assess how darcin concentration may influence neurogenesis, females were exposed to male BALB/c urine with the addition of one of a range of darcin concentrations (0.01μg/μl, 0.1μg/μl, 0.5μg/μl, 1μg/μl, 2μg/μl, 3μg/μl); females exposed to male BALB/c urine with the addition of only buffer served as '0μg/μl' controls. There was a significant positive correlation between the number of Ki67-stained proliferating cells in the SVZ and the concentration of darcin (Spearman's rho: r_s = 0.77, p < 0.001; **Figures 4.10a & 4.11**). Similarly, the number of DCX-positive immature neurons in the hippocampus was positively correlated with the concentration of darcin present in the male urine females were exposed to (Spearman's rho: r_s = 0.85, p < 0.001; **Figures 4.10b & 4.12**). Upon further inspection of the data, females exposed to male urine containing a concentration of r-darcin below the estimated threshold level had significantly fewer Ki67-stained proliferating cells in the SVZ and DCX-positive immature neurons in the hippocampus than females exposed to urine containing a concentration of r-darcin above the estimated threshold (Mann-Whitney U test: Ki67: U = 6.00, Z = -3.02, p = 0.001; DCX: U = 7.00, Z = -2.93, p = 0.001) However, the correlations observed between darcin concentration and positively stained cells were not due to a response simply based on whether urine contained a concentration of r-darcin above or below the threshold. The number of positively stained cells and darcin concentration were significantly correlated when data from only those females exposed to urine containing concentrations of r-darcin above the threshold level were assessed (Spearman's rho: Ki67: r_s = 0.71, p = 0.02; DCX: r_s = 0.84, p = 0.003).

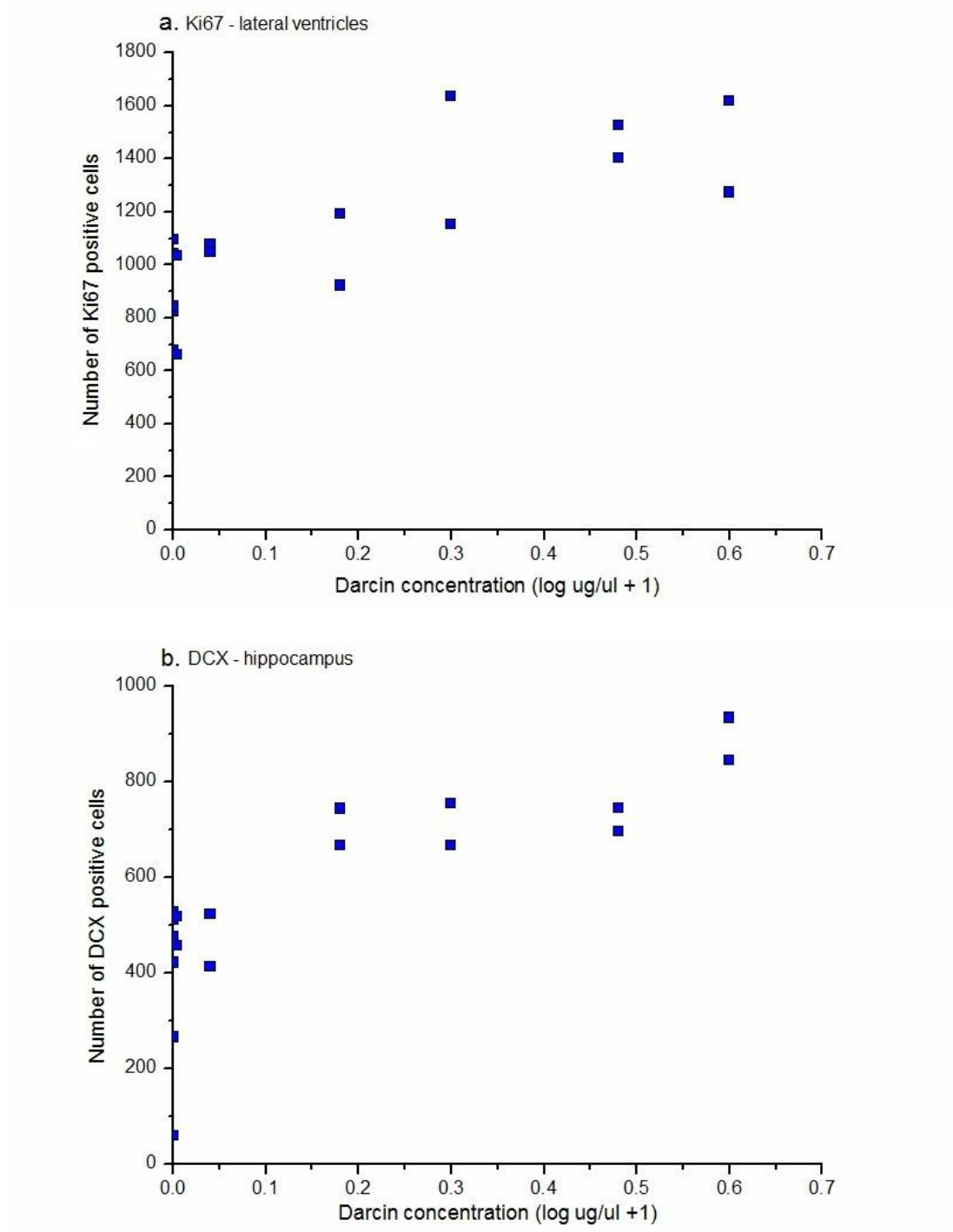

Figure 4.10 Proliferating cells and immature neurons in females exposed to male urine containing different concentration of darcin.

Number of (a) Ki67-positive proliferating cells in the SVZ and (b) DCX-positive immature neurons in the dentate gyrus of females exposed to male BALB/c urine containing 0μg/μl, 0.01μg/μl, 0.1μg/μl, 0.5μg/μl, 1μg/μl, 2μg/μl or 3μg/μl r-darcin. The number of Ki67-positive cells was positively correlated with darcin concentration (r_s = 0.77, p < 0.001). DCX-positive staining was also positively correlated with the darcin concentration of urine (r_s = 0.85, p < 0.001).

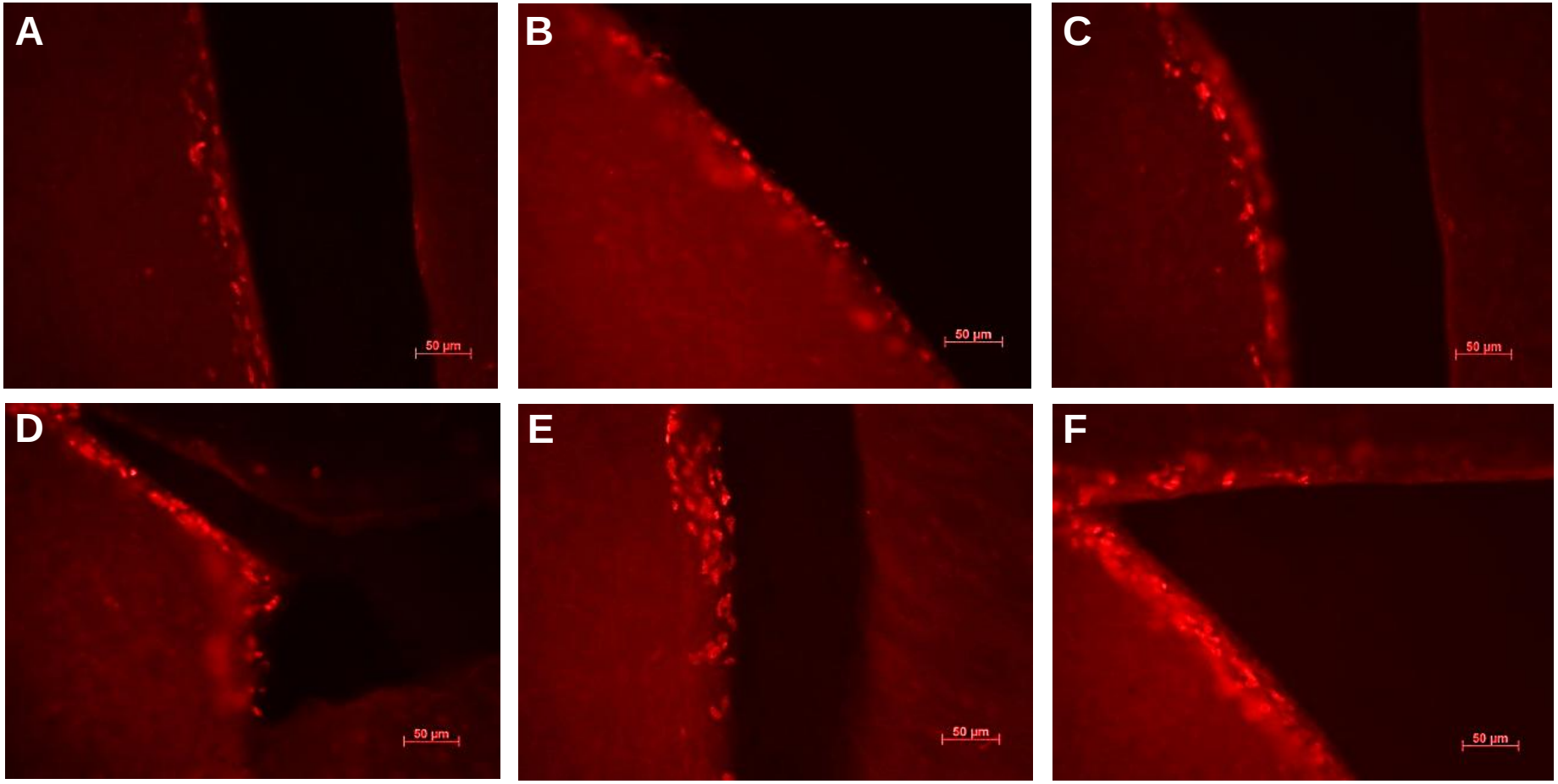

Figure 4.11 Representative micrographs of Ki67-positive cells.
Ki67-positive cells in the SVZ of the lateral ventricles in females exposed to male BALB/c urine + (A) 0.01µg/µl, (B) 0.1µg/µl, (C) 0.5µg/µl, (D) 1µg/µl, (E) 2µg/µl or (F) 3µg/µl r-darcin.

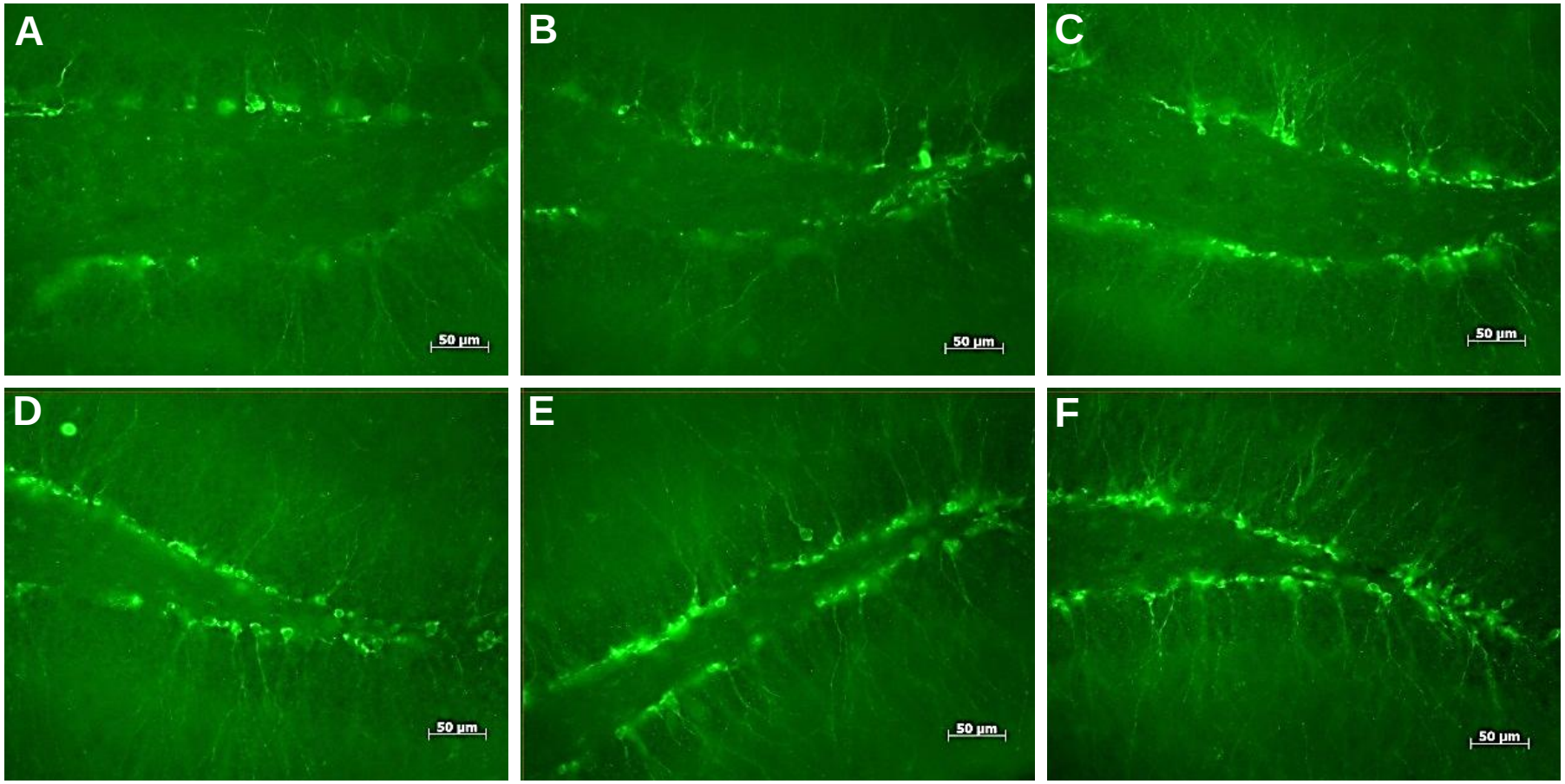

Figure 4.12 Representative micrographs of DCX-positive cells.
DCX-positive cells in the SGZ of the dentate gyrus (hippocampus) in females exposed to male BALB/c urine + (A) 0.01µg/µl, (B) 0.1µg/µl, (C) 0.5µg/µl, (D) 1µg/µl, (E) 2µg/µl or (F) 3µg/µl r-darcin.

4.3.7 Is a consistent scent signature of individual identity required for male urine to stimulate an increase in neurogenesis?

Males defend scent-marked territories and excluding other males or countermarking intruder scents informs females honestly of competitive ability (Rich and Hurst, 1999). Females are therefore more likely to come into contact repeatedly with the scent of a dominant local territory owner and familiarity may act as a proxy for quality (Cheetham *et al.,* 2008); the ratio and pattern of MUPs expressed provides an olfactory identity signature important for recognition of familiar individuals (Hurst *et al.,* 2001). Urine from male laboratory mice containing darcin is sufficient to stimulate an increase in neurogenesis, but unlike wild male urine, contains uniform expression of MUPs across individuals of each strain (Cheetham *et al.,* 2009). To establish whether consistency in the individual scent signature is important for stimulation of neurogenesis, females were exposed to urine from a single wild stock male or urine from three different wild stock males presented in succession. Compared to females exposed to male BALB/c urine, the number of Ki67-positive cells in the SVZ was significantly greater in all females exposed to wild male urine (Mann Whitney U test: single male: $U = 5.00$, $Z = -2.08$, $p = 0.02$; three different males: $U = 5.00$, $Z = -2.08$, $p = 0.02$; **Figures 4.13a & 4.14**). Further, there was no significant difference in the response to urine from one or multiple individuals. (Mann Whitney U test: $U = 16.00$, $Z = -0.32$, $p = 0.41$). However, the number of DCX-positive cells in the hippocampus was only significantly greater in females exposed to urine from a single male (Mann Whitney U test: single male: $U = 3.00$, $Z = -2.40$, $p = 0.008$; three different males: $U = 12.00$, $Z = -0.96$, $p = 0.20$; **Figures 4.13b & 4.15**); further, there was a significant difference in the number of DCX-positive cells between these groups (Mann Whitney U test: $U = 4.00$, $Z = -2.24$, $p = 0.01$).

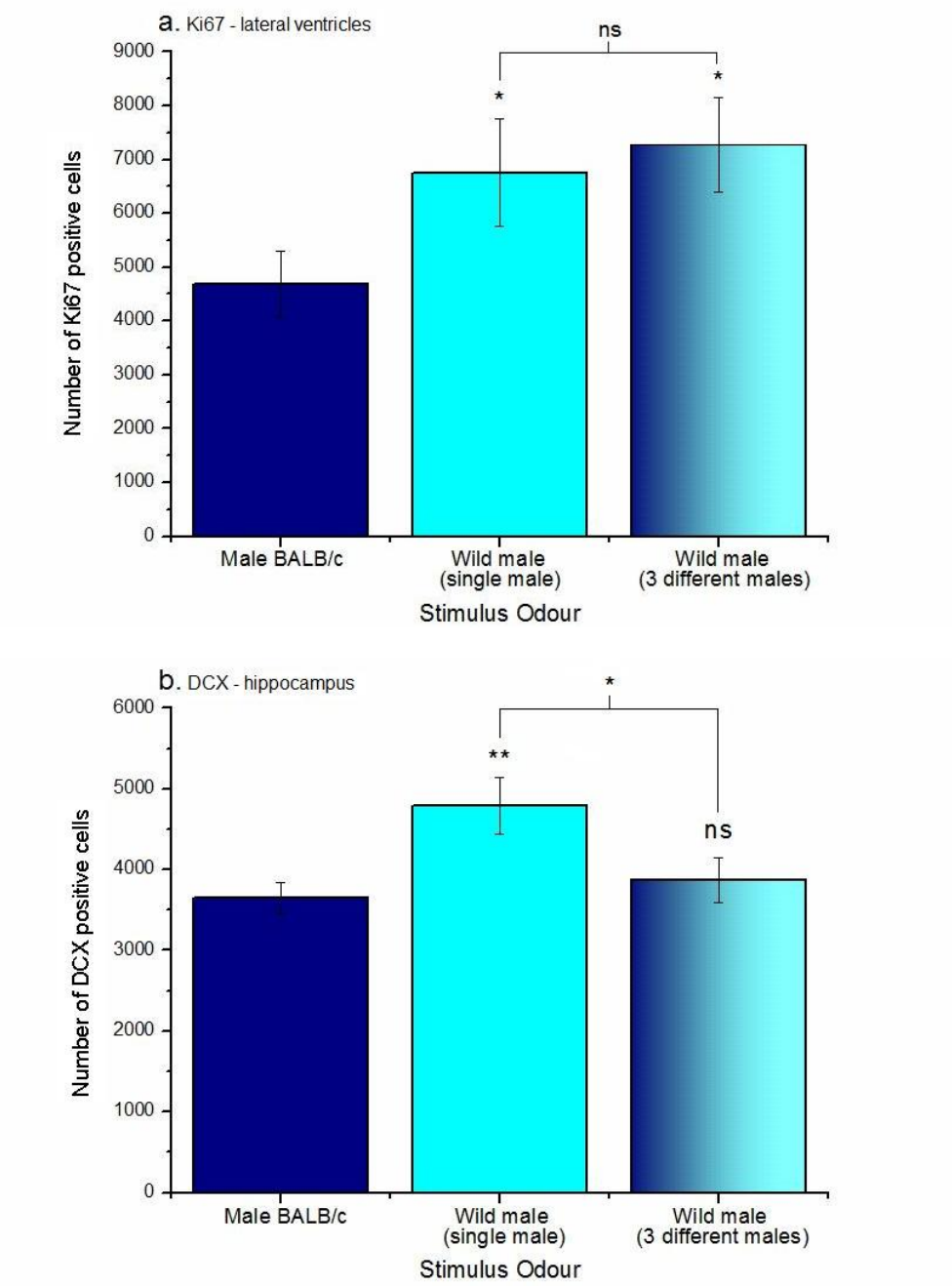

Figure 4.13 Proliferating cells and immature neurons in females exposed to urine from laboratory or wild male mice.

Number of (a) Ki67-positive proliferating cells in the SVZ and (b) DCX-positive immature neurons in the dentate gyrus of females exposed to male BALB/c urine (*dark blue*), urine from one wild male (*light blue*) or urine from three different wild males (*faded blue*). A greater number of immunopositive cells in females exposed to wild male urine versus in females exposed to a male BALB/c urine control was assessed by Mann-Whitney U tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean number of stained cells ± SEM.

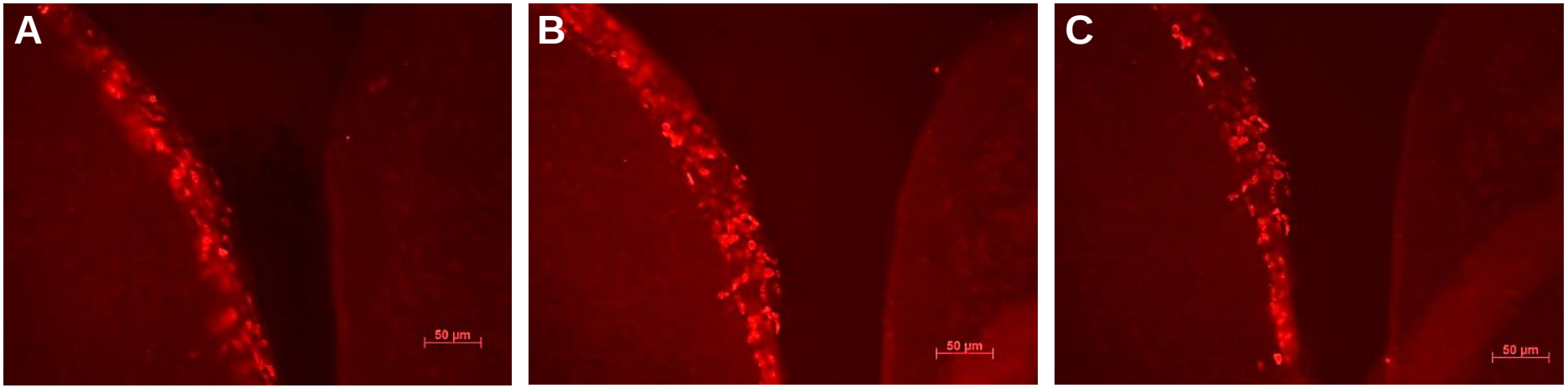

Figure 4.14 Representative micrographs of Ki67-positive cells.
Ki67-positive cells in the SVZ of the lateral ventricles in females exposed to (A) male BALB/c urine (B) urine from one single wild male or (C) urine from three different wild males.

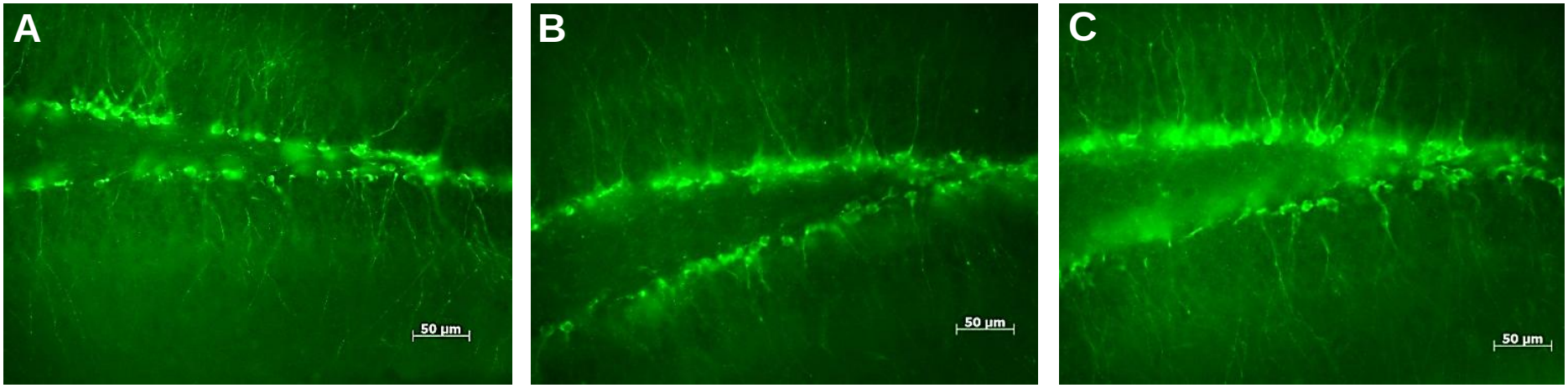

Figure 4.15 Representative micrographs of DCX-positive cells.
DCX-positive cells in the SGZ of the dentate gyrus (hippocampus) in females exposed to (A) male BALB/c urine (B) urine from one single wild male or (C) urine from three different wild males.

4.3.8 Does prolonged exposure to male scent stimulate a more robust learned preference for the location of male scent?

Male urine can condition a preference for its location following a learning session as brief as 10 minutes (Roberts *et al.,* 2012). However, following a single 10 minute scent exposure, extinction of a conditioned place preference occurs rapidly if no scent is encountered in a previously conditioned site (Roberts *et al.,* 2012). As adult hippocampal neurogenesis has been associated with processes important for long-term spatial memory (Snyder *et al.,* 2005), it may therefore be important for promoting more robust learning of male scent locations due to prevention of the extinction of a learned preference even when no scent is encountered. To investigate whether prolonged exposure to male urine over 7 days, which may stimulate an increase in neurogenesis, prevents extinction of a learned preference for the location of male scent, conditioned place preference was assessed in females presented with unfamiliar wild male urine and familiar wild male urine that females were exposed to for 7 days prior to the learning session. A separate group of females was exposed to the familiar male urine stimulus for only 30 minutes prior to the learning session to establish if responses of females following a 7 day pre-exposure were due to the length of pre-exposure and a potential increase in neurogenesis, or simply due to the effect of prior familiarity with a scent. After confirming no location bias (no urine), female subjects were presented with the urine stimuli (familiar and unfamiliar wild male urine) and two control stimuli (ddH_2O) during a single, 10-min learning session. Conditioned place preference was then tested 24 hours and 48 hours later with no urine present (24h and 48h memory).

During the learning session, there was a significant main effect of stimulus on female attraction ($F_{(2,72)} = 18.97$, $p < 0.001$; **Figure 4.16**); this was not influenced by pre-exposure time (effect of pre-exposure time: $F_{(1,36)} = 2.56$, $p = 0.12$). Contrasts revealed females showed a significant attraction to both familiar and unfamiliar urine (familiar: $F_{(1,36)} = 32.55$, $p < 0.001$; unfamiliar: $F_{(1,36)} = 27.37$, $p < 0.001$), with no significant difference in this attraction between females pre-exposed for 30 minutes and females pre-exposed for 7 days (interaction between pre-exposure time and stimulus: familiar: $F_{(1,36)} = 0.11$, $p = 0.74$; unfamiliar: $F_{(1,36)} = 0.17$, $p = 0.68$). Females were attracted to both familiar and unfamiliar male urine and this attraction did not differ according to how long females were pre-exposed to the familiar male urine.

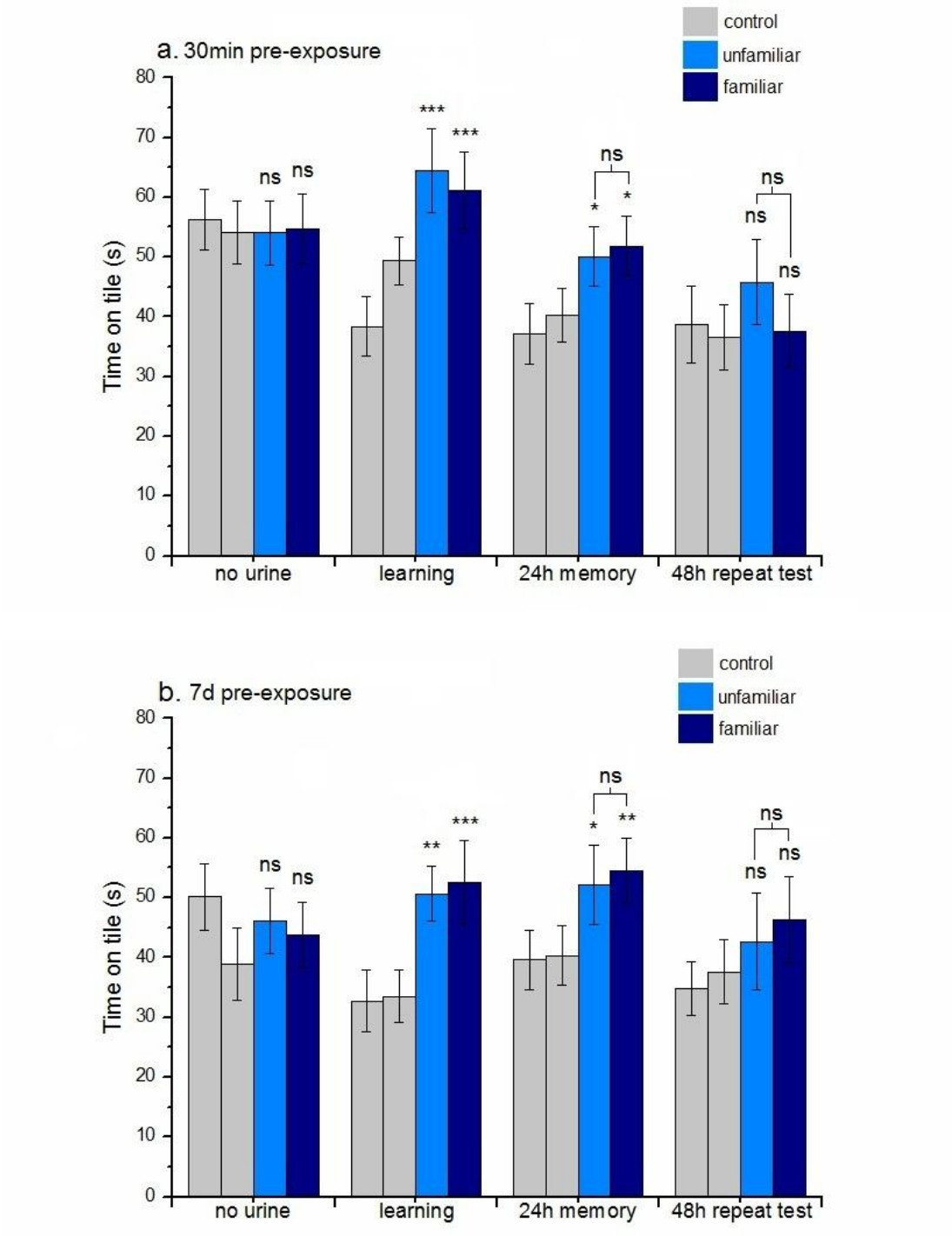

Figure 4.16 Female attraction and retention of learned spatial preferences for familiar and unfamiliar wild male urine.

Time spent by female subjects in urine or control locations when presented with unfamiliar male urine and familiar male urine females had been exposed to for (a) 30min or (b) 7d prior to the learning session. [(a) and (b) $n = 19$ subjects]. Greater time spent in male urine location versus mean time spent in control locations was assessed by separate repeated measures ANOVAs for data presented in panels a and b: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent mean ± SEM.

Similarly to responses of females during the learning session, in the subsequent conditioned place preference test, there was a significant main effect of stimulus on female learned preferences ($F_{(2,72)}$ = 11.13, p < 0.001; **Figure 4.16**); this was not influenced by pre-exposure time (effect of pre-exposure time: $F_{(1,36)}$ = 0.10, p = 0.75). Contrasts revealed females showed a learned preference for both familiar and unfamiliar urine (familiar: $F_{(1,36)}$ = 18.13, p < 0.001; unfamiliar: $F_{(1,36)}$ = 12.51, p = 0.001), with no significant difference in these learned preferences between females pre-exposed for 30 minutes and females pre-exposed for 7 days (interaction between pre-exposure time and stimulus: familiar: $F_{(1,36)}$ = 0.04, p = 0.84; unfamiliar: $F_{(1,36)}$ = 0.02, p = 0.90). This suggests females showed a learned preference for both familiar and unfamiliar male urine and that this did not differ according to how long females were pre-exposed to the familiar male urine.

When tested on a second successive day with no scent present, there was no significant effect of stimulus on the time females spent in each location ($F_{(2,72)}$ = 2.17, p = 0.12; **Figure 4.16**); this was not influenced by pre-exposure time (effect of pre-exposure time: $F_{(1,36)}$ = 0.03, p = 0.87). Contrasts revealed a conditioned preference for the locations of either familiar or unfamiliar male urine was absent (familiar: $F_{(1,36)}$ = 2.09, p = 0.16; unfamiliar: $F_{(1,36)}$ = 4.04, p = 0.062), with no significant difference in the lack of a learned preference between females pre-exposed for 30 minutes and females pre-exposed for 7 days (interaction between pre-exposure time and stimulus: familiar: $F_{(1,36)}$ = 2.12, p = 0.15; unfamiliar: $F_{(1,36)}$ = 0.06, p = 0.81). Learned spatial preferences for male urine were absent on the repeat test day. However, the response of females to the unfamiliar urine during the 48h repeat test tended towards significance, but this was not influenced by pre-exposure time. This indicates pre-exposure to male urine, and therefore prior familiarity with that scent may have altered the extinction of female learned preferences for male scent locations in this experiment; the length of pre-exposure had no significant effect on this response.

4.4 DISCUSSION

4.4.1 Stimulation of adult neurogenesis by exposure to male urine and the male-specific MUP, darcin

Neurogenesis has been implicated in a range of learning and memory tasks that require individuals to memorise and recall odours or spatial locations, and is also regulated by exposure to conspecific scents. For example, soiled bedding encompassing urine, faeces and other olfactory cues from dominant males stimulates neurogenesis in both the hippocampus and olfactory bulb, whilst odours from castrated or subordinate males do not (Mak *et al.*, 2007). As urine contains important male pheromones and is a major component used in olfactory communication in mice, the first objective of this study was to investigate whether male urine is able to stimulate a significant increase in neurogenesis in females. The results show that male urine from unrelated individuals of the same laboratory strain as the female subjects was sufficient to stimulate an increase in cellular proliferation in the SVZ of the forebrain and an increase in the number of immature neurons in the hippocampus.

Interestingly, in contrast to previous studies, an increase in hippocampal neurons of females exposed to female urine also occurred. Urine was collected from females of the same strain as subjects, so it is unlikely that novelty played a role in stimulating this increase. However, laboratory mice have been shown to discriminate the scents of individual mice of the same strain used in this experiment (Arakawa *et al.,* 2008), despite the fact that inbreeding of these strains creates almost identical patterns of MUP expression in the urine of individuals (Cheetham *et al.,* 2009). As females were exposed to urine from unfamiliar and unrelated individuals, this stimulus may have been perceived as novel, resulting in increased investigation and activation of neural pathways that could lead to an increase in neurogenesis. When presented with novel odours, there is innate motivation for individuals to investigate a novel scent more than a familiar one (Gregg and Thiessen, 1981). This may lead to an increase in initial sniffing and activation of the olfactory pathway (including the VNO) (Dulac and Torello, 2003; Luo *et al.,* 2003). Olfactory activity is associated with the level of olfactory neurogenesis (Rochefort *et al.,* 2002; Rochefort and Lledo, 2005), and disruptions to olfactory neurogenesis impair the ability of individuals to discriminate between odours (Gheusi *et al.,* 2000). However, only hippocampal neurogenesis appears to have been influenced

here, so olfactory activity is unlikely to have had a significant effect. Alternatively, individual differences in this particular group of females may be important. Although all subject females were bred, weaned, housed and handled in an identical manner, the subjects in this treatment group may have responded differently to the experimental procedure. Hormone levels and physical activity are potent regulators of hippocampal neurogenesis (Tanapat *et al.,* 1999; van Praag *et al.,* 1999), and it cannot be precluded that the oestrus status, overall baseline hormone levels or activity patterns of these animals may have influenced the results. As females were paired with siblings, it is also possible that differences in maternal care and the proportion of males present in the natal nest may have influenced neurological developmental (Bredy *et al.,* 2003). However, this is less likely as siblings from the same litter were balanced across treatment groups. It is also possible that, as a result of multiple testing, the use of one-tailed tests, and potential chance effects, type I error influenced the result obtained; the difference between the two treatment groups was concluded to be significant when in fact it may not be.

Variation between urine donors may also have influenced the result obtained. A small number of laboratory females have been shown to express the male-specific MUP, darcin, in their urine (Cheetham *et al.,* 2009). This protein is essential for female learned preferences for male scent locations (Chapter 3) and in this study was important for stimulation of an increase in proliferating cells and immature neurons. It is therefore possible that the urine collected from some of the female donors contained components not typically expressed by laboratory females of this strain. To address these issues, a second group of subjects was exposed to female urine from other unrelated and unfamiliar individuals of the same strain. No significant increase in DCX-staining was observed in these females, and when data from the two experiments were combined, overall there was no significant difference in the number of DCX-positive cells between females exposed to female urine and those exposed to water. Therefore, the original results may be a consequence of individual differences in subjects or urine donors that could not be controlled for. In future experiments, it may be useful to monitor the activity patterns or hormones of animals where possible to ensure individuals with unusual behaviour or hormone levels are removed or accounted for (e.g. Fabel and Kempermann, 2008; Schoenfeld and Gould, 2012; Galea *et al.,* 2013).

In this study as a whole there was a high level of variation in the number of positively stained cells between individual experiments. For example, the number of Ki67-poisitve cells in females exposed to male BALB/c urine varied from 900 to 4500 in different experiments and the number of DCX-positive neurons varied from 375 to 3500. Many of the factors outlined above may be responsible for this high level of variation. In addition, although breeding and handling procedures were maintained across all experiments, differences between animals in the separate experiments are likely to be present. This inherent variation between individuals in underlying behaviour or neurobiology is also magnified by the small group sizes used (Llorens-Martin *et al.,* 2006; Button *et al.,* 2013). As the study progressed, the technical skills required for conducting perfusions, tissue processing and microscopy also improved. In addition, polyclonal primary antibodies were used to detect Ki67 and DCX antigens. Whilst this improves detection of antigens that may be expressed at low levels, variability between batches of antibodies manufactured is higher. As a result, differences in detection rate and staining intensity between experiments may occur, leading to a high degree of variation in cell counts as observed.

The first experiment also aimed to establish the role of the male–specific MUP, darcin, in the stimulation of neurogenesis when females are exposed to male scent. Females were exposed to either male BALB/c urine or male BALB/c urine with the addition of r-darcin. The results show a significant increase in cellular proliferation and neuron number in females exposed to urine containing r-darcin, but not in females exposed to male BALB/c urine alone. However, the number of Ki67-positive cells in females exposed to male BALB/c urine tended towards significance when compared to the number of positively stained cells in females exposed to the control (ddH_2O). However, there was a significant difference in the number of Ki67-positive cells when females exposed to male BALB/c urine and male BALB/c urine with the addition of r-darcin was compared directly; further, in a subsequent experiment there was no significant difference in the number of Ki67-positive cells between females exposed to male BALB/c urine and females exposed to the control (ddH_2O) ($p = 0.47$). This suggests darcin must be present as a component of scent for female neurogenesis to be stimulated by exposure to male odours. Darcin is a large, involatile protein that most likely activates receptors present in the VNO after delivery via direct contact and sniffing (Luo *et al.,* 2003). The VNO is connected to the accessory olfactory bulb and

adult neurogenesis providing new neurons to this region is regulated in a similar manner to neurogenesis that provisions the main olfactory bulb. For example, aggressive behaviour in males and exposure of females to male odours increases the number of new neurons being integrated into the accessory olfactory bulb (Nunez-Parra *et al.,* 2011). Darcin may therefore be involved in the activation of specific sensory neurons that in turn elicit changes in neurogenesis.

Male odours induce oestrus in females and addition of soiled male bedding to home cages of females is a reliable method of inducing oestrus in the laboratory (Marsden and Bronson, 1964). Oestrus can also be induced in females by exposure to male MUPs (Marchlewska-Koj *et al.,* 2000). During the oestrus phase, circulating oestrogens increase to levels several times greater than during other stages of the cycle in rodents (Butcher *et al.,* 1974), potentially influencing cellular proliferation and survival. During proestrus when oestrogen levels are high, the number of proliferating cells in the DG reaches the highest level. This is in contrast to the low levels of proliferation during diestrus (Tanapat *et al.,* 1999). As male odour and male MUPs stimulate oestrus in adult females, it is possible that darcin plays a role in mediating this effect. Females exposed to male urine containing this pheromone for several days may experience an increase in circulating oestrogens during the course of the experiment. As a result, a transient, but detectable increase in hippocampal neurogenesis may have occurred. However, previous reports that oestrogens do not influence hippocampal neurogenesis in mice suggest the influence of this hormone may be species-dependent (Lagace *et al.,* 2007). As female hormone levels were not monitored during these experiments it unclear whether oestrogen levels changed significantly or influenced the changes in the number of hippocampal neurons observed. Monitoring female hormones levels during the course of odour exposure in future experiments may provide useful information on the links between the induction of oestrus by male odours and neurogenesis.

When recombinant darcin is added to male BALB/c urine the overall protein concentration is increased. As male BALB/c urine typically contains lower levels of overall protein than the urine of CD-1 males, the result observed may have been a response to the increased concentration of urinary protein. Additionally, recombinant MUPs are stored in phosphate buffer; this solution is therefore combined with male BALB/c urine when r-darcin is added. This buffer is novel to females and could

potentially influence neurogenesis. To confirm the specificity of the response to darcin, females were exposed to male BALB/c urine containing phosphate buffer or male BALB/c urine containing r-MUP7, another recombinant male-specific MUP (Mudge *et al.,* 2008; Roberts *et al.,* 2010). There was no significant increase in cellular proliferation or neuron number when females were exposed to male BALB/c urine containing buffer or r-MUP7, supporting the hypothesis that darcin specifically is important for the increase in neurogenesis observed when females are exposed to male scent. Additionally, a significant increase in the number of proliferating cells in the SVZ of the forebrain and immature neurons in the hippocampus was observed in females exposed to just recombinant darcin (i.e. not in the context of other urinary components); the activation of other receptors within the olfactory systems by other urinary components appears to not be required. An inherent neurogenic response to this single protein provides individuals with a simple but reliable mechanism for facilitating learning and memory for spatial locations of male scent.

In contrast to the results presented here, a recent study reported that females exposed to a synthetic analogue of the male volatile pheromone, thiazole, had significantly more proliferating cells in the SVZ of the forebrain compared to females exposed to water (Koyama *et al.,* 2013). Whilst darcin binds the majority of thiazole in male urine, male BALB/c urine without the addition of r-darcin does contain this volatile pheromone (Robertson *et al.,* 1993). This result therefore suggests that exposure to urine from BALB/c males should stimulate an increase in forebrain cellular proliferation; a response that was not observed in the study outlined here. However, two major differences in methodology between the study conducted by Koyama *et al* (2013) and the experiments described here may account for the difference in the data. Firstly, the technique used to present stimuli to female subjects was different. In the experiments presented here, male urine or urinary components were added to clean nesting material before being placed in the home cages of females. This allowed individuals free investigation of urine stimuli over the course of the experiment and selective close contact sniffing and delivery of scent components to the VNO. By contrast, Koyama *et al* (2013) deposited solutions containing synthetic thiazole directly onto the noses of female subjects, meaning delivery of pheromones including thiazole to the olfactory system may have occurred during normal inspiration without the need for selective investigation. This potential difference in stimulation of the olfactory

system may, in part, explain the discrepancy in the results. Secondly, the concentration of synthetic thiazole (250ppm) presented to females was much greater than the typical concentration present in laboratory male urine (1.3ppm; Jemiolo *et al.,* 1986). Consistent with olfactory system neurogenesis being mediated by the level of olfactory stimulation an animal experiences (Rochefort *et al.,* 2002), stimulation of neurogenesis by this volatile component may only occur when very high concentrations are delivered to the olfactory system.

4.4.2 The influence of urinary darcin concentration on stimulation of neurogenesis in females

The next aim of this study was to establish whether darcin concentration influences the magnitude of the increase in neurogenesis observed when females are exposed to male scent. When females were exposed to male urine containing a range of concentrations of darcin, the results showed a significant correlation between darcin concentration and the number of proliferating cells and immature neurons. This suggests stimulation of neurogenesis in females is not only regulated by the presence of darcin, but the extent to which neurogenesis increases is influenced by the concentration of darcin in male scent. In some species, the amount of a specific pheromone released influences the strength of the innate response of conspecifics (Jones and Hamilton, 1998; Sumpter and Beekman, 2003; Martin and Lopez, 2010), and in mice, as protein production is likely to be metabolically costly (Gosling *et al.,* 2000), the amount of darcin present in a scent may indicate male quality as only high quality males would be able to withstand the costs associated with high levels of protein expression. MUP production is also under multi-hormonal control and male-specific MUPs such as darcin are androgen dependent (Knopf *et al.,* 1983). As testosterone is often associated with dominance, aggression and the ability of males to defend their territory (Zielinski and Vandenbergh, 1993), darcin levels may reflect subtle underlying individual differences in testosterone levels, thus indicating competitive ability. As a result, a stronger olfactory and spatial memory for scent containing higher levels of darcin may be beneficial to females.

The mechanism behind this neurogenic response may be linked to the high concentration of darcin stimulating females to investigate a scent to a greater extent,

increasing delivery of this protein to the VNO during sniffing (Luo *et al.,* 2003). The amount of darcin may therefore govern how attractive a scent is, the amount of sniffing during scent investigation, and subsequently the amount of pheromone delivered to VNO receptors. An increase in delivery of darcin to VNO receptors could explain the change in Ki67-staining as the concentration of darcin is increased; proliferation in the forebrain supplies the olfactory bulbs with new neurons and olfactory activity strongly influences the rate at which proliferation occurs (Rochefort *et al.,* 2002). It is unclear whether an increase in the number of responding receptors or an increase in the rate at which receptors respond is important. However, the activation specificity of neuronal receptors in the VNO suggests only a subset of neurons would respond to this specific protein (Matsuoko *et al.,* 1999; Punta *et al.,* 2002). As a result, an increased activation rate of VNO neurons could stimulate increased activity in the accessory olfactory bulbs and higher order processing centres of the brain, ultimately leading to an increase in newly generated hippocampal neurons.

4.4.3 The importance of direct contact with male scent for stimulation of neurogenesis

To address the importance of direct contact with scent for stimulation of neurogenesis, females were exposed to male BALB/c urine containing r-darcin that they were able to contact, or male BALB/c urine containing r-darcin that they were unable to contact. The results show only a significant increase in proliferating cells and immature neurons in females able to directly contact the urine. Without direct contact, females would not be able to deliver darcin to the VNO receptors despite stimulation of the main olfactory epithelium by other associated volatile components of male scent. However, these components of male scent alone do not appear to be sufficient to stimulate neurogenesis, even when darcin is present, unless females are exposed to concentrations over 100 times greater than is normally present in male urine (Koyama *et al.,* 2013). This is consistent with other behavioural responses to male scents, which must be contacted for learning of the associated volatile signature to occur (Ramm *et al.,* 2008; Roberts *et al.,* 2010); females must also make contact with a scent in order to form a remembered preference for the scent location (Roberts *et al.,* 2012). This suggests a requirement of accessory olfactory system processing and activation of the receptors

present in the VNO for both the behavioural and neurological changes in response to male scent.

4.4.4 The importance of a consistent scent signal of identity for stimulation of neurogenesis

To addresses whether the individuality signal within male scent must remain consistent during odour exposure for neurogenesis to be stimulated, females were exposed to urine from a single wild male or urine from three different males presented consecutively. Wild male mice were chosen as urine stimulus donors for this experiment as individual variation in the expression of components that signal individuality (e.g. MUPs and MHC-type) is extremely limited in laboratory mice due to generations of inbreeding. By contrast, the urine of individual wild males comprises different ratios and patterns of MUPs that provide a signature of individuality and genetic identity (Hurst *et al.,* 2001; Cheetham, *et al.,* 2007). By presenting females with urine from a single individual wild male, females were exposed to scent containing a consistent signal of individuality for seven days; females exposed to urine from three different males experienced male scent constantly, but the scent signature of individual identity changed. The results showed that the number of Ki67-positive cells was significantly greater in females in both treatment groups compared to in females exposed to male BALB/c urine. This suggests the rate of cellular proliferation in the forebrain is increased by exposure to wild male scent independent of the individuality signature within scent. However, the number of DCX-positive cells in the hippocampus only increased in females consistently exposed to urine from a single male. This suggests the individual male scent signature must be consistent during odour exposure for an increase to hippocampal neurons to occur; hippocampal neurogenesis may be dependent upon the consistency of the individuality signal present in male scent during exposure.

The distinct result obtained in the two regions could due to the fact that Ki67 is a marker of cell cycle activity and as a result only labels newly proliferating cells. By contrast, DCX is associated with the early stages of neuronal differentiation and immature neurons. This means conclusions with respect to neurogenesis occurring specially in the forebrain cannot be made. However, the rate at which new cells are

generated in the area that provisions the olfactory system is influenced by male scent regardless of the underlying scent signature of individual identity. Repeated exposure to the same scent results in habituation and decreased investigation, but olfactory receptors would be stimulated even when sniffing is minimal; seven days of exposure to the urine of laboratory males is sufficient to stimulate an increase in proliferating cells in the forebrain. Darcin is also able to stimulate cellular proliferation, suggesting this male-specific pheromone regulates proliferation in the forebrain regardless of whether the individuality signature in scent females are exposed to is consistent. Urine of wild male mice therefore has the same stimulatory effect on forebrain cell generation as laboratory male urine.

Hippocampal neuron number, however, was dependent upon the consistency of the male scent signature that females were exposed to. Male urine as a general stimulus was therefore not sufficient to stimulate an increase in immature neurons present in the hippocampus. The specificity of receptors in the main and accessory olfactory organs suggests scent components that signal individual identity, such as MUPs, may stimulate a specific subset of receptors. The activation of a consistent pattern of receptors during the period of scent exposure may therefore be required for an increase in neurons to occur. This result is surprising given the presence of darcin in the odour stimuli females were presented with. The result does not however contradict the conclusion that darcin is required and sufficient for stimulation of adult neurogenesis; rather, it suggests the regulation of neuron survival in the hippocampus is under the control of a much more complex mechanism.

Females prefer the owners of familiar scents over novel ones (Cheetham, 2006), and the presence of an individual male's scent in a consistent location over time is an important indicator of his competitively ability to defend a territory (Rich and Hurst, 1998). The presence of a scent that is consistent in identity and spatial location is therefore informative to both competitors and potential mates (Rich and Hurst, 1999). Responding appropriately to inconsistency in scent signatures in a given location may be important in mate choice decisions. In addition, individual differences in social status signalled through scent influence the rate of female neurogenesis (Mak *et al.,* 2007), suggesting this process may be important for discriminating between males of differing social status. The results of this experiment suggest that neurogenesis may play a role in finer discrimination between males in terms of individual identity. This may allow

females to identify and recognise specific individuals and respond appropriately to the consistency of a scent signature in a given location. However, only regulation of adult neurogenesis has been addressed here; the functional relevance of this process for specific mate choice behaviour remains unclear.

The differences in Ki67 and DCX staining in this final experiment are indicative of one of the main limitations of this study. Neither of these is an exclusive marker covering all stages of neurogenesis. Ki67 only labels proliferating cells, whilst DCX labels immature neurons and the neurons counted here would not be fully functional or integrated into the hippocampal circuits. As a result, data presented here do not provide conclusive evidence of increases in functional neurogenesis as a result of prolonged exposure to male scent. Conclusive evidence of neurogenesis would require fate-tracking of cells, which can be done via quantification of cells labelled with an exogenous thymidine analogue such as BrdU and other endogenous markers such as NeuN. This could be developed in future work, enabling a clearer picture of the stimulation of neurogenesis by male scent to be developed.

4.4.5 The role of neurogenesis in female learning of male scent locations

Results presented here suggest that darcin is important for the regulation of adult female neurogenesis. This pheromone is also important for female attraction to male scent and in conditioning a remembered preference for male scent locations (Roberts *et al.,* 2012). However, the mechanism by which paired odour and spatial memories are formed may not be reliant upon neurogenesis. The neurogenic response in females is time dependent; two days of exposure does not stimulate an increase in neurogenesis whilst seven days is sufficient to stimulate an increase in cellular proliferation and new immature neurons (Mak *et al.,* 2007). Despite this requirement for prolonged exposure, females are able to form remembered preferences and spatial memories of scent locations in periods as brief as ten minutes. Therefore, neurogenesis is unlikely to be important in immediate learning of male scent locations, as only brief contact with a scent is required for females to assess and learn the locations of preferred male scents.

To investigate whether improvements in the robustness of female learning occur as a result of prolonged exposure to male urine over 7 days, the extinction of a learned preference for the location of male scent was utilised as a measure of learning robustness. This was assessed in females presented with unfamiliar wild male urine and familiar wild male urine simultaneously in a conditioned place preference test. Females were exposed to the familiar male urine for 7 days prior to the learning session; a separate group of females was exposed to a familiar male urine stimulus for only 30 minutes prior to the learning session to establish if responses of females following a 7 day pre-exposure were due to the length of pre-exposure and a potential increase in neurogenesis, or simply due to the effect of prior familiarity with a scent. However, as a result of 7 days of scent pre-exposure, cell proliferation and the number of immature neurons may increase, but the process of neurogenesis would not be complete. As a result, this experiment was not able to directly assess how the complete process of neurogenesis influences extinction of learned spatial preferences in females. Additionally, the robustness of female learning was only assessed with regards to the reversal of an extinction of a learned spatial preference. It is possible that other measures of learning robustness, such as the length of time the learned spatial preference remains following the learning session, may be influenced more effectively by an increase in neurogenesis.

The results showed that prior prolonged exposure to male urine did not create more robust spatial learning of male scent locations; a conditioned place preference was absent on the repeat test day regardless of whether pre-exposure to male urine lasted for 30 minutes or 7 days. Despite this, the response of females to the location of unfamiliar male urine on the repeat test day tended towards significance, suggesting pre-exposure to male urine may influence a more robust response to the sites of male scents in general. This result may simply be due to chance effects; when the responses of females pre-exposed to male urine for either 30 minutes or 7 days were assessed separately, neither group showed a learned preference for the location of male urine during the 48 hour repeat test. As this tendency was not influenced by the length of pre-exposure, simply being familiar with a scent may be more important. This suggests that the stimulation of adult neurogenesis in females associated with prolonged exposure to scent may not play a role in enhancing the robustness of learning of male scent locations. However, during the pre-exposure period, male urine was added to nesting

material in the home cage of female subjects. Females then encountered this familiar urine in a specific location outside of the home cage within the context of a conditioned place preference test. This mismatch in the location in which urine was presented to females may have obscured the effects of urine-stimulated neurogenesis on spatial learning; prolonged exposure to male urine in a specific site may be required for more robust learning of male scent locations, providing a memory for accurate navigation guided by the familiar male territory owner's scent. Alternatively, urine-stimulated neurogenesis may be important for other learning processes such as the development of more long-term memory for male scent locations. Further conditioned place preference tests examining extended memory of male scent locations, or assessment of female learning of nest sites where repeated exposure to male scent occurs, may be useful in identifying the importance of neurogenesis stimulated by male scent for combined spatial and olfactory learning.

Further work is also needed to identify the functional relevance of increases in neurogenesis in response to darcin. In this study, only the regulation of neurogenesis could be addressed. Blocking this process via chemicals or irradiation would be required for the functional relevance of darcin-stimulated neurogenesis to be determined. It is unlikely that learning of scent locations in conditioned place preference tests would be disrupted due to the brief time period required for these learned preferences to be formed. However, the importance of neurogenesis for more robust spatial and olfactory memories should be tested as it seems appropriate that some aspect of spatial memory should rely on this process. Blocking neurogenesis removes the standard preference for a dominant male over a subordinate individual (Mak *et al.,* 2007), so it is possible that more fine scale individual discrimination is disrupted when olfactory neurogenesis is blocked. The male-specific pheromone darcin is important for a number of behaviours including learning of individual scent signatures and induction of spatial memories of scent locations. Olfactory and hippocampal neurogenesis are also stimulated by this male-specific pheromone. This reliable and consistent behavioural response of females to darcin, combined with the reliable neurological response, suggests it is fundamental to regulating responses to conspecific scents in mice.

CHAPTER 5: General discussion

5.1 GENERAL DISCUSSION

The overall aims of this thesis comprised three main components. The first was to assess whether male investment in ultrasonic calls and scent marks in mice is dependent upon the social context in which signalling is measured, and to investigate several other factors that may mediate individual investment in these sexual signals. The second aim was to evaluate the importance of male scent signals and components such as darcin for female learning and behaviour; specifically, the learned spatial preferences of females for multiple locations of competing male scent marks were assessed. Finally, the third aim was to determine whether the same scent signals that stimulate female spatial learning also stimulate adult neurogenesis, by quantifying hippocampal neurons and newly generated cells in the forebrain of female mice exposed to male scents or scent components. Overall, experiments combined observation of sexual signalling in different social contexts, conditioned place preference tests to assess female learning in response to male scent cues and neurological studies using immunohistochemistry to link male signalling investment, female learning and adult neurogenesis.

5.1.1 Male Signalling investment

The overall aim of the two experiments described in Chapter 2 was to investigate why male mice invest in multiple sexual signals and what physiological or social factors might influence male signal investment. In the first experiment, investment in scent marking and calling was recorded in a range of contexts to address why males produce two different sexual signals and how investment according to social context may be linked to individual testosterone levels. The results of this experiment showed investment in both calling and scent marking differed according to social context; time spent calling was greatest during interactions and scent marking was greatest in response to female odours. This is consistent with previous hypotheses regarding the importance of calls for courtship and the role of scent marks in providing long lasting olfactory signals in the environment that are important for attraction of females (Nyby, 1983; Hurst *et al.,* 1998; Hurst, 2009). The result is also consistent with the multiple messages hypothesis, which describes how the use of multiple male signals may evolve in response to a need for investment in different signals for communication in different contexts (Tinbergen, 1959; Moller and Pomiankowski, 1992). In mice, males

may invest in one signal to attract females from a distance and a second signal during close-contact sexual encounters. For males, this could reduce time and energy costs associated with high investment in both signals at the same time. Females may also reduce their costs associated with close inspection of all potential mates; by utilising scent marks to first assess males when the scent owner is absent, females could selectively approach only those males whose scent signals are preferred and further assess male quality based on calling investment (Candolin, 2003).

Data collected in the first experiment showed that individual testosterone levels were only linked to signalling investment under specific conditions, suggesting that situational and hormonal factors may interact to mediate expression of sexual signals. As testosterone was only correlated with signal investment when both scent marking and calling were relatively high, it is possible that this hormone only influences signalling when males are pressed to invest at the highest levels of an individual's ability or range. However, due to the fact that multiple tests were conducted to assess the relationship between testosterone and scent marking or calling in multiple contexts, this result must be interpreted with caution. Additionally, testosterone may have a more consistent influence on other aspects of calling not measured during this experiment, such as pitch, call syllables and formants (e.g. Beani et al., 1995; Cynx *et al.,* 2005; Evans *et al.,* 2008). The social context in which testosterone was linked to signalling investment was also the context in which urinary protein levels were correlated with scent marking (i.e. when males were separated from females by a barrier). However, further analysis revealed that the number of scent marks deposited was negatively correlated with urinary creatinine concentration, a measure of urinary dilution. This suggests the number of scent marks produced by males may partially have been mediated by the volume of urine in the bladder when marks were deposited. These results highlight the importance of taking into account the context in which signalling is quantified when assessing the influence of a variety of factors on individual investment, particularly in species that produce different signals in different contexts.

The final objective of the first experiment was to identify any relationships between male signalling and the behaviour of males and females during sexual interactions. As calling is thought to be important for courtship in mice, it was hypothesised that male motivation would be positively correlated with calling. The results showed that calling was positively correlated with the level of male affiliative

behaviour, but contrary to expectations, males that mounted the least typically called the most. However, these males also displayed the most affiliative behaviour, suggesting there were important individual differences in mating strategies between males (Rolland *et al.,* 2003). The lack of a relationship between calling and female behaviour was unexpected. However, previous studies suggesting female affiliative behaviour is influenced by male calls measured simple approach behaviour or the time spent near a tethered male (Pomerantz *et al.,* 1983; Hammerschmidt *et al.,* 2009), thus, the evidence for a significant effect of male calling on female behaviour is limited. It is possible that once a female has initially approached a male, call rate does not influence subsequent behaviour. Alternatively, other characteristics of male calls that were not measured here may be more important for female preferences (e.g. Reid *et al.,* 2004; Wyman *et al.,* 2012). Despite investment in scent marks being low during interactions compared to other contexts, the level of affiliative behaviour displayed by females was negatively correlated with the number of scent marks males deposited. Males that scent mark at a high rate may be more competitive and more likely to aggressively defend a territory from unfamiliar individuals. As a result, females may have been more cautious and avoided prolonged direct contact with these males during interactions. This may have been intensified by the absence of prior contact with male scent marks before interactions, and thus the opportunity for females to be selective about which males to interact with. This lack of prior contact with scent may also have led females to investigate scent marks more than would normally occur during interactions, stressing the importance of these olfactory signals for female behaviour and mate choice, even in the context of courtship.

The second experiment was conducted to extend the findings of interaction trials in the first experiment. As a result of single sexual interactions in experiment one, only correlations between male signalling investment and the behaviour of males and females could be identified. This limited identification of causality in this relationship (i.e. does female behaviour influence male signalling investment or does male signalling influence the female behavioural response?). In experiment two, subjects participated in multiple sexual interactions to establish whether male investment in signalling is a stable trait or may be influenced by the behaviour of different individual females. Additionally, the influence of another hormone, corticosterone, on male investment in sexual signals was examined; evidence suggests this hormone produced as a component of the stress

response may influence sexual signalling and behaviour (Moore and Miller, 1984; Retana-Marquez *et al.,* 1996; Buchanan, 2000).

The investment of individual males in scent marking and calling was consistent relative to other males across multiple interactions; further, scent marking did not differ significantly when overall investment was compared across the three sexual interactions. However, male calling increased during the course of the experiment, suggesting all males increased their call rate but by similar amounts, leading to minimal changes in male ranks. This suggests male signal investment within the context of social interactions is likely to be a relatively stable and consistent trait, consistent with previous findings regarding investment in this signal (Whitney *et al.,* 1973). Calling investment of all males may increase following experience with a female, regardless of female behaviour or male motivation. Alternatively, as all females used in these experiments were from the same inbred laboratory strain, although males interacted with three different females, they may have perceived each female as the same individual, and thus responded with a similar level of signal investment during each interaction.

During the first experiment, the behaviour of males was linked with signal investment, and males that typically mounted the most and displayed the least affiliative behaviour showed the lowest investment in calling. By contrast, during the second experiment, despite male calling increasing over the course of this experiment, affiliative behaviour declined and more mounting attempts were made, although the two were not correlated. This change in behaviour is consistent with previous studies showing how male sexual behaviour changes with experience, regardless of the female a male is paired with (Coopersmith *et al.,* 1986; Swaney *et al.,* 2012). Further, the stability of male signalling relative to other individuals and the significant decline in affiliative behaviour of both males and females suggests that male investment in scent marks and calls is not strongly influenced by individual differences in the sexual motivation or behaviour of females. Additionally, as scent marks are likely to be more important for attracting females rather than as a signal during courtship, male investment may not differ across multiple interactions as it is not the primary signal required.

Male calling in the second experiment was positively correlated with sperm count, potentially indicating that call rate can provide females with a consistent, reliable signal of male quality. As females may first assess males based on the pattern, rate and composition of scent marks and subsequently choose to interact with preferred

individual scent owners, it would be beneficial for male calling investment to indicate some further aspect of quality not indicated by scent marks. However, during this experiment, the reproductive success of individual males was not assessed; thus, the relationship between sperm count and the number or quality of offspring females could produce as a result of choosing males that call at a high rate is unclear. It is also unclear whether females actually choose between males based on call rate, as previously only the associative preferences of females have been assessed when presented with a male that either calls or does not (e.g. Pomerantz *et al.,* 1983).

Finally, the results of the second experiment revealed that seminal vesicle weight was negatively correlated with the percentage of urinary darcin relative to other MUPs. This suggests, as a result of sexual encounters, male may balance strategies for success in pre- versus post-copulatory competition (e.g. Danielsson 2001; Pizzari *et al.,* 2002; Klaus *et al.,* 2011). Darcin is a male-specific MUP important for female attraction to male scent and for female spatial learning of the locations of male scent (Roberts *et al.,* 2012). By contrast, proteins produced in the seminal vesicles are important in sperm competition and in the formation of copulatory plugs (Bradshaw and Wolfe, 1977). The result obtained in this experiment suggests that in the context of sexual interactions, when investment in seminal vesicle proteins important for post-copulatory competition was high, the percentage of urinary MUP expressed as darcin, a protein important for pre-copulatory selection, was low. Whether males can alter this investment according to the context in which they encounter females or female signals is unknown, but further work assessing seminal vesicle gland size and darcin investment in other contexts could potentially provide evidence for this.

Overall, the results of these two experiments indicate that many factors influence male signal investment, but that no single factor is likely to mediate investment alone, particularly in a system where multiple signals are utilised in different social contexts. Hormone levels, social context and the costs associated with expression of specific signal components may all interact to mediate investment in signals important for attraction of females and courtship. More specifically, the results show that male investment in darcin may change in response to social encounters, suggesting males may invest differentially in this protein according to changing social pressures. Despite the influence of several factors across multiple social contexts, within one context, male signalling is relatively stable, potentially providing females with a

consistent, reliable indicator of male quality. Further, scent marking appeared to be linked to female behaviour, even during interactions, highlighting the importance of these olfactory signals.

5.1.2 Male scent and female spatial learning

When addressing the influence of many factors on sexual communication, it is important to assess changes in behaviour of both the signaller and the receiver. In Chapter 2, the behaviour of male signallers was addressed with regards to the factors that influence signal investment. Results of experiments outlined in Chapter 2 are consistent with previous hypotheses regarding the function of male scent marks in attraction of females. In Chapter 3, the response of females to male scent marks was assessed in the context of female learning of male scent locations. The overall aims of this chapter were twofold: firstly, the factors that influence female learning of single male scent locations were assessed; secondly, as females would encounter multiple scents when investigating male territories in a natural context, how females respond according to the comparative composition of multiple scents in a competitive context was assessed.

The results of tests conducted to assess female responses to single male scents revealed that female laboratory mice form learned preferences for the locations of male, but not female scents. Further, there was a significant effect of darcin on the learned spatial preferences of female laboratory mice; spatial learning was only observed in response to male urine containing darcin. This is consistent with the responses of wild female mice in similar tests (Roberts *et al.,* 2012), suggesting that despite inbreeding over many generations and a reduction in variability of scent mark signal components across individuals of each strain (Cheetham *et al.* 2009), laboratory females respond to male scents in much the same way as wild mice. When female learning of male scent locations was assessed in the context of competitive signalling, females showed learned preferences for the locations of multiple male scents when the scents were equivalent. Further tests revealed that, even when presented with multiple male scents, females only formed learned preferences for the locations of male scents containing darcin, whether naturally produced or added to urine as a recombinant protein. This shows, as expected, that female learned preferences for multiple male scent locations are also mediated by

darcin. However, the response of females is highly specific to the precise location of darcin and does not generalise to the location of other scents nearby that are identical except for the presence of darcin. Induction of learning of male scent locations by darcin provides females with a simple mechanism to locate the sites of preferred scents previously encountered.

The ability of this protein presented on its own (i.e. not in combination with other urine components) to stimulate a response in females was also tested; this protein stimulated female learning, and the responses of females to recombinant darcin versus recombinant darcin added to male urine were not different. This indicates darcin is as potent a stimulator of female learning as male urine containing this recombinant protein. These results are consistent with previous work showing that darcin stimulates female learning in wild mice (Roberts *et al.,* 2012). This MUP is male-specific and naturally produced by all wild males, allowing females to immediately target the most informative scents for recognition and assessment of potential mates. The pheromone also provides a mechanism for learning the volatile olfactory signature of a scent (Roberts *et al.,* 2010), meaning females may only need to remember the approximate location of a preferred scent; the recognised volatile signature could then guide females to the exact location. This would save time and energy in the search for a preferred male.

Females show a learned preference of the location of male urine containing approximately 1μg/μl darcin, but not male urine containing negligible levels of naturally produced darcin. It was therefore hypothesised that the threshold concentration for a learning response must lie somewhere between these two quantities. To establish what the threshold concentration for a response is, females were exposed to numerous male scents containing different quantities of added recombinant darcin. Females responded to urine containing 0.1μg/μl darcin but not 0.05μg/μl darcin. This result suggests that females only form a learned preference for male urine if it contains a minimum concentration of between 0.05μg/μl and 0.1μg/μl darcin. This is approximately five times lower than the lowest concentration found in wild stock male urine samples analysed in this study. All the males sampled had higher concentrations of urinary darcin than would be required to stimulate learned preferences in females; further, these males were singly housed and expression is likely to be even greater under competitive conditions in the wild (Garratt *et al.,* 2012). This suggests that competitive expression of

darcin may be important to males, despite extreme costs that may be associated with the production of high quantities of this scent mark component (Gosling *et al.,* 2000).

This analysis lead to the hypothesis that males may produce quantities of darcin much greater than the minimum needed to stimulate spatial learning in females in order to be competitive against other males in attracting females and securing mating opportunities. Thus, the amount of darcin in a scent may influence learned preferences when scents containing different concentrations representative of typical expression by males are encountered simultaneously by females. To test this, in place preference tests comprising four stimuli, females were presented with male urine containing a relatively low concentration of darcin and urine containing a relatively high concentration of darcin during a single learning session. In one test, the difference between the low and high concentrations was 1μg/μl and in a separate test the difference between the low and high concentrations was 2μg/μl. This allowed not only the effect of darcin concentration on female responses to be assessed, but also whether the relative amount of darcin is important when females are comparing multiple male scents. The results showed learned spatial preferences of females differed significantly in response to the scent containing a high concentration of darcin compared to the scent containing a low concentration of darcin. In addition, there was no effect of the size of the difference in concentration between the scents; females showed a stronger learned preference for the location of the scent containing a high concentration of darcin regardless of the difference in concentration between the scents. This suggests that in the context of competitive scent marking, females may compare male scents based on the concentration of darcin and form stronger learned preferences for the scent containing the highest concentration. This is consistent with other work assessing the influence of pheromone concentration on female responses to males (e.g. Phelan and Baker, 1986; Dussourd *et al.,* 1991), and identifies what may be a particularly important component for competitive scent signalling and female choice.

Further tests revealed that differences between competing scents according to other characteristics are also important. For example, when presented simultaneously, females only formed a learned spatial preference for a familiar scent when the other scent was novel. This suggests that whilst darcin mediates female spatial learning of male scent locations and must be present in a scent for this response to occur, other factors influence female learning of male scent locations. This allows the learned

preferences of females for male scent locations to be both inherent and selective, providing an important innate response to male urine in general, but a response that can be influenced by other factors such as familiarity and pheromone concentration.

The presence of darcin in male scent underlies the ability of females to form remembered preferences for the locations where they encounter male scent, representing an important mechanism by which spatial learning can occur in a natural context. The darcin pheromone was able to condition place preference, suggesting it may be a particularly significant social signal for the attraction and learning of female mice in response to multiple competing male scents in specific locations. The ability of darcin to induce learning adds a further function to the repertoire of this pheromone; it also mediates learning of the volatile odours associated with the specific scent of an individual male (Roberts *et al.,* 2010). This suggests a potentially important new role for pheromones in stimulating more than one type of associative learned response – to both the specific volatile profile of a scent and to the spatial location of scents.

5.1.3 The influence of male scent on female neurogenesis

Results described in Chapter 3 show that the immediate behavioural learning response of females to male scent signals is mediated by the male specific MUP, darcin. As the formation of learned spatial preferences is likely to require a combination of both olfactory and hippocampal processing, male scent signals may influence the physiology of these brain regions. Neurogenesis in the olfactory system and hippocampus has been shown to be influenced in females by exposure to male odours (Mak *et al.,* 2007); the aim of experiments presented in Chapter 4 was to assess whether longer term neurobiological changes that occur as a result of exposure to male scent are mediated by the same scent mark components that stimulate learning of male scent locations.

The first objective was to confirm that male urine can stimulate neurogenesis in the olfactory system and hippocampus of the female brain. In previous studies showing the neurogenic response of the female brain to male odour, soiled male bedding or intact males were used as stimuli (Mak *et al.,* 2007; Larsen *et al.,* 2008). It was therefore important to first establish whether one of the components of male scent that stimulates this response is in urine. The results showed that cellular proliferation in the

SVZ and neuron number in the DG was increased in females exposed to male urine, suggesting the component of male scent that stimulates neurogenesis is in urine. Next, females were exposed to urine from male laboratory mice that excrete negligible levels of naturally produced darcin (BALB/c); this urine was presented to females alone or with the addition of recombinant darcin at a concentration equivalent to what is known to stimulate spatial learning. This allowed assessment of whether the same urinary protein that mediates an immediate spatial learning response to male scent also mediates a more long term neurological change. The results showed that darcin is required for stimulation of adult neurogenesis in females exposed to male urine.

Further tests revealed that this response was due to darcin specifically. Cellular proliferation and the number of hippocampal neurons did not increase in females exposed to male urine containing another recombinant MUP. This suggests the presence of novel buffer or an overall increase in protein that occurs when recombinant darcin is added to male urine did not influence the neurogenic response. Results of further experiments in this study also showed that direct contact must be made with male urine for neurogenesis to be stimulated, suggesting that the bound volatile components of darcin cannot mediate the response alone and that contact with the actual protein itself is important. This highlights the importance of the involatile protein itself for stimulation of adult neurogenesis and not simply the fact that it binds another volatile pheromone, extending its release (Armstrong *et al.,* 2005). However, another recently published study suggested that thiazole, the volatile component bound by darcin, is able to stimulate an increase in cell proliferation in the SVZ of the forebrain when females are exposed to very high concentrations of this scent component (Koyama *et al.,* 2013). It is possible that this volatile component can only stimulate an increase in cell proliferation at concentrations much greater than would typically be present in male urine, explaining the different results presented in the study outlined here and in the study conducted by Koyama *et al.* (2013). As the concentration of darcin in male urine was important for female learning as described in Chapter 3, the influence of darcin concentration on neurogenesis was also tested. The results showed that not only does darcin mediate neurogenesis in response to male scent, but the extent to which an increase in neurogenesis occurs is influenced by the concentration of darcin present in male urine.

The mechanism that mediates this neurogenic response may be due to darcin stimulating females to investigate a scent and increased delivery of this protein to the VNO during sniffing (Luo *et al.*. 2003). The amount of darcin may therefore govern how attractive a scent is, the amount of sniffing, and subsequently the amount of pheromone delivered to VNO receptors. An increase in delivery of darcin to VNO receptors could explain the change in Ki67-staining as the concentration of darcin is increased; proliferation in the forebrain supplies the olfactory bulbs with new neurons and olfactory activity strongly influences the rate at which proliferation occurs (Rochefort *et al.,* 2002). Further, VNO neurons maintain a constant current showing slow adaptation after firing, but an increase in firing rate occurs as the strength of the stimulus increases (Liman, 1996). As a result, increased activation rate of VNO neurons could stimulate increased activity in accessory olfactory bulb and higher order processing centres of the brain, ultimately leading to an increase in immature hippocampal neurons.

A final experiment revealed that neurogenesis in females exposed to male scents that contain either a consistent or changing olfactory signature of identity had differential changes in neurogenesis in the SVZ and the hippocampus. When females were exposed to urine from a single wild male for the duration of the experiment, both cellular proliferation in the SVZ and neuron number in the DG increased. By contrast, when exposed to urine from three different wild males in succession, cellular proliferation increased in the forebrain but neuron number in the hippocampus did not. Two important factors may have influenced these results, either independently or in combination. Firstly, neurogenesis in the lateral ventricles, which supplies new neurons to the olfactory bulbs, may be stimulated differentially to neurogenesis in the hippocampus. As previous results presented in Chapter 4 indicate that darcin is required for male scent to stimulate neurogenesis, it is possible that delivery of darcin to the VNO and stimulation of receptors that may respond to this protein is all that is required for the rate of olfactory system neurogenesis to increase, regardless of the background olfactory signature of identity. By contrast, a constant change in the individual scent signature may alter how higher order centres of the brain, including the hippocampus, process the overall scent signal. As a result, the change in scent signature that occurred at regular intervals during the experiment would eliminate the prolonged period of time required for upstream processing to stimulate hippocampal neurogenesis. Secondly, cellular proliferation was measured in the forebrain and neuron number was quantified

in the hippocampus. This suggests consistency in MUP pattern may only be important for one specific stage of neurogenesis – cell selection. The delivery of darcin to the olfactory system and the resulting processing that occurs in higher order brain structure may stimulate an overall increase in proliferation, regardless of the background scent signature of identity. However, once cells reach the selection stage of neurogenesis prior to differentiation, a consistent signal of individuality may be required for cellular survival as one of the many factors that influence survival rates (Dayer *et al.,* 2003).

Overall, the results of experiments presented in Chapter 4 indicate that, as in female spatial learning, darcin mediates neurogenesis in females exposed to male scent. Further, other factors may influence the extent to which neurogenesis is stimulated, such as the concentration of darcin. However, how other factors influence the extent to which neurogenesis is stimulated may depend upon the stage of neurogenesis being quantified or the region of the brain in which this process is measured. Despite the importance of the connections between the olfactory system and the hippocampus for processing sensory information, this highlights the fact that, ultimately, they are separate regions of the brain and may respond differently according to their more specific roles in learning and processing information (Jarrard, 1995; Brennan and Keverne, 1997; Lledo *et al.,* 2005). In addition, the importance of measuring more than one marker of neurogenesis may be important in any future experimental work, as studies of cellular proliferation and selection may provide differing results.

Finally, the conditioned place preference tests presented in Chapter 4 indicate that pre-exposure time (either 30 minutes or 7 days) had no significant effect on the learning responses of females; prolonged exposure to male scent and the potential stimulation of neurogenesis may not be important for enhancing the measure of learning robustness quantified in this study. However, females used in these place preference tests were older than those used in the other experiments quantifying neurogenesis in response to urine stimuli. As a result, the effects of prolonged scent exposure may have been minimal compared to those presented in the earlier experiments due to age-related declines in overall levels of neurogenesis (Ben Abdallah *et al.,* 2010). Alternatively, it is possible that an increase in neurogenesis associated with prolonged scent exposure is not important for female learning of scent locations; rather, it has a different, unidentified role in learning (reviewed by Deng *et al*., 2010).

5.2 CONCLUSIONS

Overall, the results of the studies presented in this thesis suggest that many factors mediate male signalling investment, and that the resulting differences in investment influence female responses to male signals, in terms of both immediate behavioural learning and long-term neurological change. Data presented in Chapters 3 and 4 shows that individual investment in a specific component of male scent marks, darcin, mediates not only female spatial learning of scent locations, but stimulation of neurogenesis associated with prolonged exposure to male scent. Further, data presented in Chapters 3 and 4 are consistent with those presented in Chapter 2; whilst one specific signal component may mediate both short-term behavioural and long-term physiological responses in females, other factors mediate the strength of the response. This reflects the importance of investigating more than one factor that may influence male signal investment or the responses of females to these signals; multiple factors must be taken into account when studying sexual communication.

The results presented in this thesis also demonstrate the behavioural and neurobiological responses to darcin that add further functions to the repertoire of this pheromone. In addition to stimulating more than one type of associative learned response (i.e. to both the specific volatile profile of a scent and to the spatial location of a scent), it also influences upstream neurological changes that may occur as a result of learning (Gould *et al.,* 1999). The fact that both behavioural and neurological responses are reliable, but behavioural responses are rapid, whilst neurological responses occurs over a more protracted period, provides a potentially important model that could be used to investigate how changes in neurobiology are linked to the olfactory processing of, and subsequent response to, important social odours.

5.3 FUTURE WORK

The results of experiments presented in Chapter 2 raise many additional questions that could be addressed in future work. One of the most important hypotheses formed as a result of data collected during the experiments outlined in Chapter 2 is that male calling investment in mice may be linked with individual sperm count. It is clear that scent marks provide females with valuable information regarding

individual male quality as part of mate choice (reviewed by Hurst, 2009). However, the importance of calling investment beyond simply reinforcing close contact or enhancing courtship is unclear. Whilst recent studies have suggested the repertoires of males are different to one another and may serve an important role in kin recognition (Holy and Guo, 2005; Musolf *et al.,* 2010), it is possible that call rate serves an additional purpose in informing females of male quality. The results of the second experiment described in Chapter 2 showed that call rate may indicate fertility and could therefore be an important indicator of male quality and potential reproductive success. To confirm the importance of calling as an important signal of male quality, two questions would need to be addressed. Firstly, do females choose between potential mates based on male call rate during sexual encounters? This would allow female choice based on this signal to be assessed; any quality indicated by this signal must be recognisable by females and be influential in female choice. Secondly, does call rate predict male reproductive success? Assessing the links between male call rates and the number or quality of offspring produced would provide important support for sperm count accurately indicating fertility and male reproductive success.

Results presented in all three data chapters raise further questions regarding male investment in the specific scent mark component, darcin. In the context of social interactions, males may balance their investment in this protein with other proteins important for post-copulatory competition. Further, the variation in expression of this protein is high across males and influences both female learning and the increase in neurogenesis as a result of exposure to male urine. It is already known that males increase production of MUPs in response to competition (Garratt *et al.,* 2012), but it would be interesting to assess whether, under normal conditions, expression of darcin is stable or whether long term changes in expression of this protein occur. Darcin expression levels of multiple males could be monitored over a prolonged period under single housing conditions or in response to sexual interactions and mating. It would also be useful to know if males increase darcin expression when only female odours are encountered; males produced the most scent marks in response to female odours during the first experiment presented in Chapter 2. It is possible that under these conditions, expression of this protein may be altered. Assessing female choice of potential mates in response to the darcin concentration of male scent marks may also be important in understanding whether this component of scent is a sexually selected signal in males; in

the studies presented here, only attraction and learned preferences for male urine stimuli were assessed.

The results of conditioned place preference tests in Chapter 3 also raise further questions. The concentration of darcin presented in male urine was important for female responses to multiple male scents that contained different concentrations, regardless of the size of the difference between the scents. In Chapter 2, results indicated males adjusted their investment according to the percentage of this protein. The percentage of darcin relative to other MUPs in urine may therefore also be important for the female response to male scent signals in the context of female learning, so assessing the importance of darcin concentration, percentage and the combination of these factors could be addressed. For example, if females are presented with two male scents that both contain a concentration of darcin equal to 2μg/μl, but in one scent this represents 15% of overall MUP and in the other scent this represents 25% of overall MUP, does this influence female learned preferences for these scents? Answering this question would provide valuable information regarding how males might adjust their investment in this component of scent marks, and whether when attempting to attract females, protein investment should be balanced across individual MUPs or males should simply aim to produce the greatest concentration of darcin possible.

Results presented in Chapter 3 also indicated that whilst darcin was required for female learning, other factors influenced the strength of the learning response. For example, females showed a learned preference for familiar male scent but not unfamiliar male scent when presented simultaneously. Given more time, it would have been useful to conduct further conditioned place preference tests to assess which of these factors is the most important for stimulating female learning of male scent locations. For example, if a familiar male scent contains a lower concentration of darcin than a novel male scent, is familiarity or darcin concentration more influential in subsequent learned preferences of females? Answering this question is essential to advancing understanding of female responses to complex male scents in a natural context. It is unlikely females would encounter male scent marks that differ simply in familiarity or darcin concentration, but are identical in all other aspects; multiple scents from different males would differ in all of these characteristics simultaneously. Thus, assessing the combined role of these factors in female learning is important.

Finally, the link between learning and neurogenesis could not be resolved by experiments presented in this thesis. The same scent mark components stimulate both female learning and neurogenesis, suggesting there may be an important link between behaviour and neurobiology in female mate choice. However, the results of the final experiment presented in this thesis indicated that neurogenesis is not important for more robust learning of male scent locations. It is possible that the stimulation of female neurogenesis by exposure to male scent containing darcin is important for other aspects of female behaviour related to mate choice. For example, neurogenesis may stimulate more long-term learning; when females are pre-exposed to a male scent for a period long enough to stimulate neurogenesis, they may be able to retain a learned preference for that scent location longer than the 14 days previously observed in female mice (Roberts *et al.,* 2012). Assessing the links between learning and neurogenesis could be achieved by investigating the functional relevance of neurogenesis for female learning of male scent locations. Blocking neurogenesis removes the normal preference of females for a dominant male (Mak *et al.,* 2007); it is possible that blocking neurogenesis may also impair the responses of females to male scents containing darcin or preferences for familiar males. Alternatively, neurogenesis may be more important for other aspects of female behaviour, such as orientation within a home range using male scent marks as stable, long-term markers of territorial boundaries and safe sites important for nesting. Ultimately, although darcin stimulates female learning of male scent locations and increases in neurogenesis, the links between changes in neurobiology and behaviour remain unclear. Further studies of the functional relevance of this process and understanding how it may influence learning may provide valuable answers to this question.

CHAPTER 6 - References

6.1 REFERENCES

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